AUG 2 3 1945

Medical Library

VOL. 30 AUGUST, 1945 No. 8

The Journal of Laboratory and Clinical Medicine

Clinical and Experimental

A Pathologic Study of Tsutsugamushi Disease (Scrub Typhus) With Notes on Clinicopathologic Correlation. Major Emmett B. Settle, Medical Corps, Army of the United States, Henry Pinkerton, M.D., St. Louis, Mo., and Major Austin J. Corbett, Medical Corps, Army of the United States 639

Conglutination Test for Rh Sensitization. Alexander S. Wiener, A.B., M.D., Brooklyn, N. Y. 662

The Detection of Rh Sensitization: Evaluation of Tests for Rh Antibodies. Louis K. Diamond, M.D., Boston, Mass., and Neva M. Abelson, M.D., Philadelphia, Pa. 668

Studies in Hodgkin's Syndrome. III. The Relationship of Tubercle Bacilli to Hodgkin's Syndrome. Herman A. Hoster, M.D., Charles A. Doan, M.D., and Mariellen Schumacher, Columbus, Ohio 675

Studies in Hodgkin's Syndrome. IV. The Therapeutic Use of Radioactive Phosphorus. Herman A. Hoster, M.D., and Charles A. Doan, M.D., Columbus, Ohio. With the Technical Assistance of Mariellen Schumacher 678

The Relationship Between Diet and the Mechanisms for Defense Against Bacterial Infections in Rats. L. Joe Berry, Ph.D., Jane Davis, B.S., and Tom D. Spies, M.D., Birmingham, Ala. 684

Treatment of Experimentally Induced Type I Pneumococcus Pneumonia in Albino Rats. Elmer H. Loughlin, M.D., Richard H. Bennett, M.D., Julius Wolf, M.D., and Mary E. Flanagan, B.S., Brooklyn, N. Y. 695

The Action of β-Dimethylaminoethyl Benzilate Hydrochloride. A Synthetic Antispasmodic. Henry M. Lee, Ph.D., Charles C. Scott, Ph.D., M.D., and K. K. Chen, Ph.D., M.D., Indianapolis, Ind. 700

(Contents continued on inside cover)

PUBLISHED BY THE C. V. MOSBY COMPANY, 3207 WASHINGTON BLVD., ST. LOUIS 3

Copyright 1945 By The C. V. Mosby Company

CONTENTS—(Cont'd)

The Significance of Very Low Values for Blood Urea Nitrogen. Russel O. Bowman, Ph.D., Frances F. Crowell, B.A., and Laura F. Olson, B.A., Providence, R. I. ---------- 706

Laboratory Methods

A Protective Cabinet for Infectious Disease Laboratories. C. C. Shepard, C. W. May, and N. H. Topping, Bethesda, Md. ---------- 712

Double-Tube Method for Isolation of Motile Bacteria. Lieutenant Colonel Max Levine and Major Paul W. Preisler, Sanitary Corps, Army of the United States ---------- 716

Determination of Amino Nitrogen of Blood Filtrates by the Copper Method. Anthony A. Albanese, Ph.D., and Virginia Irby, B.S., Baltimore, Md. ------ 718

Book Reviews

Book Reviews ---------- 722

Editor

CARL V. MOORE, M.D.
Washington University School of Medicine
600 South Kingshighway
St. Louis 10, Mo.

Editorial Board

CLIFFORD J. BARBORKA, M.D.
Northwestern University, Chicago

PAUL R. CANNON, M.D.
University of Chicago, Chicago

CARL D. CLARKE, Ph.D.
University of Maryland, Baltimore

RUSSELL L. HADEN, M.D.
Cleveland Clinic, Cleveland

GEORGE HERRMANN, M.D.
University of Texas, Galveston

DENNIS E. JACKSON, M.D.
University of Cincinnati, Cincinnati

JOHN A. KOLMER, M.D.
Temple University, Philadelphia

W. C. MacCARTY, M.D.
Mayo Clinic, Rochester, Minn.

T. B. MAGATH, M.D.
Mayo Clinic, Rochester, Minn.

EDWARD MUNTWYLER, Ph.D.
Long Island College of Medicine, Brooklyn

VICTOR C. MYERS, Ph.D.
Western Reserve University, Cleveland

M. H. SOULE, Sc.D.
University of Michigan, Ann Arbor

GERALD B. WEBB, M.D.
Colorado Springs, Colo.

Vol. 30, No. 8, August, 1945. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis (3), Mo. Subscription Price: United States, its Possessions, Pan-American Countries, $8.50 per year; Canada, $9.50; Foreign, $9.50. Entered as Second-Class Matter at Post Office, at St. Louis, Mo., under Act of March 3, 1879. Printed in the U. S. A.

A PATHOLOGIC STUDY OF TSUTSUGAMUSHI DISEASE (SCRUB TYPHUS) WITH NOTES ON CLINICOPATHOLOGIC CORRELATION

MAJOR EMMETT B. SETTLE, MEDICAL CORPS, ARMY OF THE UNITED STATES, HENRY PINKERTON, M.D., ST. LOUIS, MO., AND MAJOR AUSTIN J. CORBETT, MEDICAL CORPS, ARMY OF THE UNITED STATES

INTRODUCTION

THE known rickettsial diseases of man excluding the still moot problem of trench fever fall into four distinct groups: typhus, spotted fever, tsutsugamushi disease, and Q fever. Typhus is carried by lice and fleas, spotted fever by ticks, and tsutsugamushi disease by mites. The rickettsiae of Q fever are found in ticks, but epidemiologic studies are incomplete. Two distinct varieties of typhus, murine and human, are recognized and strain differences, notably in virulence, are found between individual members of the other three groups. The existence of complete cross-immunity between all varieties and strains within each of these groups, however, and the invariable absence of cross-immunity between members of different groups, are ample justification for regarding the classification as definitive.

Scrub typhus has become the commonly employed clinical designation of a rickettsial disease which recently has assumed importance because of its occurrence among troops in New Guinea and in adjacent islands of the Southwest Pacific area. The careful work of Lewthwaite and Savoor[1] has shown that this disease is essentially identical clinically, pathologically, and immunologically with tsutsugamushi disease. The term scrub typhus, therefore, should be regarded as having only temporary value and must eventually be discarded in the interest of nosological clarity, just as the term Sao Paulo typhus has been abandoned in favor of (Brazilian) spotted fever now that the disease is known to be identical with Rocky Mountain spotted fever.

Scrub typhus certainly is no more closely allied to the typhus group than to the spotted fever group of diseases, and there is every reason for separating it sharply from both. This purpose would be achieved most simply by calling it tsutsugamushi disease. Any strain differences which may come to light as a result of study could be indicated by names analogous to murine typhus and human typhus. In this way confusion in terminology and perhaps unwarranted assumptions regarding therapeutic indications could be avoided.

For a general discussion of tsutsugamushi disease, including the clinical picture, diagnosis, and epidemiology, reference is made to recent papers by Alhm and Lipshutz[2] and Farner and Katsampes.[3] These authors express a preference for the name tsutsugamushi disease.

Historical Orientation.—Tsutsugamushi disease has been much less thoroughly studied, in its etiologic and pathologic aspects, than have typhus and

Based upon work carried out in the Department of Pathology, Third Medical Laboratory, stationed overseas, and in the Department of Pathology, St. Louis University School of Medicine.

Received for publication, May 17, 1945.

spotted fever. Japanese investigators in 1928[4] and 1931[5] demonstrated the etiologic agent in the testicle of the rabbit and in the anterior chamber of the rabbit's eye, propagated the agent by these rather inconvenient methods of inoculation, and named it *Rickettsia tsutsugamushi* (*orientalis*) (*nipponica*). Lewthwaite and Savoor,[6] in a single instance, isolated a strain in guinea pigs rendered vitamin deficient. In the course of recent work, including ours, the isolation and propagation of strains have been accomplished repeatedly by the intraperitoneal injection of human blood into white mice and guinea pigs. This has greatly facilitated the laboratory study of the disease. The repeated isolation of strains in this manner, in contrast to previous failures, can be explained perhaps as a phenomenon of strain variation. The reaction of the guinea pig to these recently isolated strains is apparently identical with that described by Lewthwaite and Savoor in their single success.

The histopathology of diseases of the tsutsugamushi group has heretofore been described only briefly.[7-9]

Objectives of the Present Study.—Our study is based on an analysis of fifty-five fatal cases of tsutsugamushi disease occurring in American troops in British and Dutch New Guinea and adjacent islands. A considerable number of experimental animals (mice and guinea pigs) reacting to strains isolated from these patients were available also for study. The purposes of the study are (1) to describe in detail the pathologic changes associated with the disease; (2) to compare these changes with those found in fatal cases of typhus and spotted fever; and (3) to determine whether an analysis of these data would bring out pathologic or physiologic differences between tsutsugamushi and the other two groups of rickettsial disease which might act as a guide to clinical research.

THE ETIOLOGIC AGENT

Our observations on the etiologic agent of the disease (*R. tsutsugamushi*) will be confined largely to its morphology, since detailed biologic and immunologic studies by others will soon be available.

The direct demonstration of rickettsiae in human tissues has been achieved only in film preparations from pericardial, pleural, and peritoneal fluid. Here they are present in much smaller numbers than in similar preparations from infected mice and guinea pigs and usually are found only after long search. Thus far we have not been successful in finding definite rickettsiae in paraffin sections of human tissues. On the basis of experience with typhus tissues, we have no reason for stating that rickettsiae are more difficult to demonstrate in tsutsugamushi than in typhus material. In spotted fever, however, rickettsiae are somewhat easier to demonstrate.

The presence of rickettsiae in smears from the serous surfaces of mice, guinea pigs, and human beings, their demonstration by Japanese workers in the testicle and in the anterior chamber of the eye,[4, 5] and their cultivation in tissue culture without loss of virulence[10] leave no serious doubt concerning their etiologic significance in tsutsugamushi disease.

Morphologically, *R. tsutsugamushi,* as seen in film preparations from the peritoneum of the mouse, certainly does not appear to differ greatly from *Rickettsia prowazeki* and *Dermacentroxenus rickettsi,* the agents of typhus and spotted fever, respectively. In all three, the predominating form is a minute diplobacillus. In size, *R. tsutsugamushi* is indistinguishable from *R. prowazeki* and also from *D. rickettsi* in its more common forms.[11] Forms resembling the large lanceolate diplococci, which occasionally are seen in spotted fever, were not

observed by us. We found that bipolar and diphtheroid forms were more common than in either typhus or spotted fever; however, since identical forms have been seen in large numbers in the brain of typhus-infected mice and occasionally in typhus-infected yolk sacs, this observation seems to be of doubtful differential value. Long chains and thread forms were not seen in our material, but such forms are found only under unusual conditions.

Cells packed and distended with hundreds of rickettsiae, so commonly seen in films from the peritoneum of experimental animals reacting to murine typhus or to human typhus, were not found in this study. Individual cells contained from ten to sixty organisms, suggesting the picture seen in smears from experimental spotted fever in guinea pigs, but the organisms tended to form loose clusters rather than being diffusely scattered as in spotted fever.

Spotted fever is characterized by the presence of rickettsiae not only in endothelial cells, but also in smooth muscle cells of arterioles, while in typhus, organisms are seen only in intimal endothelial cells.[11] A comparison of tsutsugamushi with typhus and spotted fever from this point of view cannot be made until suitable material is available.

The presence of rickettsiae in nuclei, a feature of spotted fever but not of typhus, can be studied only in sections and is rarely demonstrable in mammalian tissues.[11] The material in which they can best be demonstrated is the infected chorioalantoic membrane of the fertile egg,[12] which was not available in this study.

PATHOLOGY

The general similarity of the pathologic picture to that of typhus and spotted fever[14] became apparent early in the course of this study. In all three diseases, the pathologic changes undoubtedly are direct results of the multiplication of rickettsiae within the endothelial cells or smooth muscle cells of capillaries, arterioles, venules, and less often of larger vessels. Swelling and proliferation of the intimal endothelium, cellular infiltration of the wall of the larger vessels, perivascular accumulation of mononuclear cells, and occasional thrombosis and hemorrhage are the essential features of the reaction. These changes are most noticeable in the skin, brain, and myocardium but are present to some extent in nearly every organ, so that the disease may be characterized as an acute generalized inflammation of the vascular lining, the vascular wall, and the perivascular tissue.

Gross Observations.—In general, the gross changes seen post mortem are not striking. The most distinctive finding upon external examination of the body is the local primary lesion at the site of inoculation by the bite of the larval mite. It is a cutaneous ulceration, varying from 2 to 15 mm. in diameter, usually covered with a blackish crust, the so-called eschar, which is only slightly adherent to a shallow grayish-pink, granular crater, with sharply defined margins. The skin surrounding the ulcer is moderately red. In our series, the primary lesion, when secondarily infected by pyogenic organisms, was often enlarged and presented a dirty gray surface with purulent drainage. Not infrequently the ulcer was in a healing stage and relatively inconspicuous. The primary lesion was observed at autopsy in 89 per cent of our cases, occurring in the locations listed in order of frequency: trunk, 32 per cent; axilla, 22 per cent; external genitals, 12 per cent; thigh, 12 per cent; lower leg, 10 per cent; inguinal region, 8 per cent; upper extremity, 2 per cent; neck, 2 per cent. In one case, two typical primary ulcers with eschars were present, one on the abdomen and the other on the thigh.

The *skin rash* observed clinically fades very rapidly after death and was present in only two of our patients upon both of whom autopsy was performed immediately after death on the twelfth day of the disease. The lesions consisted of confluent, red, mottled macules from 1 to 2 cm. in diameter scattered over the trunk, upper arms, and thighs.

Necrotic lesions of the skin were absent and hemorrhagic lesions were most unusual, appearing in but two of our series. On one patient there was an area of purpura 8 cm. in diameter over the sacrum, accompanied by recent petechial hemorrhages over the arms and hands, which appeared terminally on the thirteenth day after an extreme elevation of temperature to 108° F. In the second patient, many recent petechial hemorrhages were scattered over the skin of the chest and face. These also appeared a few hours before death on the thirteenth day of the disease.

The *serous cavities* commonly presented characteristic gross changes. Free fluid was often encountered which was cloudy and straw colored and contained small flecks of whitish-yellow exudate. The serous surfaces tended to be slightly dull and injected, occasionally studded with punctate hemorrhages.

In 56 per cent of our cases the cloudy straw-colored fluid in the pericardial sac was increased, although not enough to cause cardiac embarrassment, as it averaged less than 100 c.c. The petechiae were most frequently located on the posterior surface of the sac.

The pleural cavities presented a similar appearance with free fluid in one or both sacs in 53 per cent of our cases, the amount ranging from 30 to 500 c.c. in 76 per cent, being over 500 c.c. in 24 per cent. In at least one-half of our cases this effusion was not associated with bronchopneumonia, and subsequent microscopic studies made it obvious that the condition represented a rickettsial pleuritis rather than a secondary bacterial infection.

A slight to moderate increase of peritoneal fluid was observed in only one-fifth of our cases.

The *heart* exhibited relatively mild changes and was of normal weight. The myocardium at times was slightly flabby and, in a few cases, contained minute, focal, pale, brownish-gray areas of degeneration or, more rarely, small recent focal hemorrhages. The endocardium was generally smooth, pale, and glistening; petechiae were rare. Valvular damage attributable to rickettsial infection was not seen.

The *aorta, vena cava, and other large vessels* were altogether without gross changes attributable to rickettsial infection. In one case, however, we observed thrombosis of the right adrenal vein and its tributaries and of a branch of the deep circumflex iliac vein associated with massive hemorrhage into the left rectus muscle, the left psoas, and left retroperitoneal space.

The *lungs* varied greatly in appearance. Usually hemorrhage and congestion of the entire organ were the constant and striking features in the cases uncomplicated by secondary pneumonia. Weight increase was moderate to marked, and the tissue was only partially crepitant. The cut surfaces were blotchy dull red to deep purple and exuded copious dark bloody fluid. Small multiple hemorrhagic infarcts occurred in six of our patients. The mucosa of the tracheobronchial tree was deeply injected and the lumen was partially filled with sticky blood-tinged mucus. The pulmonary picture closely resembled that of the hemorrhagic pneumonitis of influenza. Less often congestion and edema were present without the hemorrhagic changes. When secondary bronchopneu-

monia was superimposed, the characteristic picture was partly masked. Microscopic examination in all cases revealed essential changes which will be described later.

The *spleen* was characteristically enlarged, firm and dull, and slaty blue. Flecks of fibrinous exudate were usually scattered over its capsule. The enlargement ranged from 300 to 700 Gm. in 65 per cent of the cases and from 200 to 300 Gm. in 28 per cent. The organ weighed over 700 Gm. in two cases, while in two others the weight was normal. Cut surfaces were deep purplish red; the splenic follicles were not prominent; the pulp was considerably softened. Focal areas of hemorrhage were fairly common and infarcts, occasionally multiple and sometimes large, were present in four cases.

Lymphadenopathy involving particularly the mesenteric, retroperitoneal, tracheobronchial, axillary, and inguinal groups was observed in approximately two-thirds of our cases. The glands were discrete, mildly to moderately swollen, succulent, and soft; the cut surfaces were pink with occasional areas of focal hemorrhage and necrosis. As a rule the lymph nodes in the region of the primary ulcer were more enlarged and areas of focal hemorrhage and necrosis more frequent. When the ulcer was secondarily infected, the lymph nodes commonly were purulent.

The *liver* had undergone no distinctive gross changes. Mild hepatic enlargement was present in 58 per cent of the series, and small, subcapsular hemorrhages were occasionally seen. The cut surface usually had a dull, cloudy appearance. The biliary tree showed no abnormality.

The *gastrointestinal tract* was without characteristic gross features, although usually punctate mucosal hemorrhages were scattered throughout its entire length. In one instance petechial hemorrhages were countless and a large amount of blood filled the entire tract from stomach to rectum; in rare cases the Peyer's patches were mildly swollen. Small focal hemorrhages were occasionally present in the serosal coats.

The *pancreas* showed no significant changes.

The *suprarenal glands* appeared normal, except in one case in which marked hemorrhage into the parenchyma had resulted from thrombosis of the adrenal vein and its tributaries.

The *kidneys* were usually swollen and congested. On the cut surfaces the dusky red pyramids stood out sharply against the pale opaque cortical parenchyma. Small infarcts were present in two cases.

The *bladder* contained occasional small submucosal hemorrhages.

The *testicles* and the *bone marrow* appeared grossly normal.

The *brain* rarely showed distinctive gross changes. The vessels of the leptomeninges were often congested. Mild cerebral edema was occasionally evidenced by slight flattening of the convolutions and gyri, usually with concomitant increase in the brain weight. In a few cases minute pinpoint hemorrhages were apparent on the cut surfaces of the brain, frequently in the brain stem and pons, rarely in the basal ganglia, the cerebrum, and the cerebellum.

The spinal fluid at times was slightly increased in amount, and occasionally was cloudy.

Microscopic Observations.—The heart was most seriously involved since the dominant lesion in all cases was acute nonsuppurative myocarditis, focal as well as diffuse, and varying in severity. This inflammatory reaction was patchy in distribution and usually most severe in the interventricular septum and the left ventricle. It was characterized by perivascular infiltration of mononuclear

cells, edema, and cellular infiltration of the larger fibrous septa, and also by a more patchy interstitial edema and cellular infiltration.

The most marked and constant finding was perivascular infiltration of mononuclear cells, chiefly plasma cells with fewer large mononuclear cells, occasional lymphocytes, and polymorphonuclear leucocytes (Fig. 1) and sometimes large multinucleated cells with vesicular nuclei and basophilic cytoplasm. This reaction occurred about vessels of all sizes but more abundantly about the smaller arterioles, venules, and capillaries. Frequently the vascular endothelium was swollen or thickened by proliferation, usually asymmetrically. The intima occasionally presented a frayed-out appearance; leucocytes often adhered to the lining, and more rarely, recent mural thrombi overlay the injured

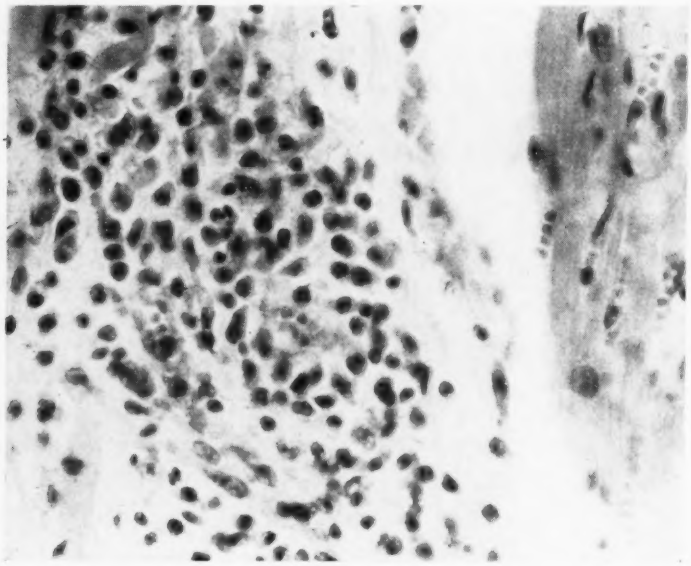


Fig. 1.—Perivascular mononuclear cell infiltration centering around a small damaged vessel in a fibrous septum in the myocardium.

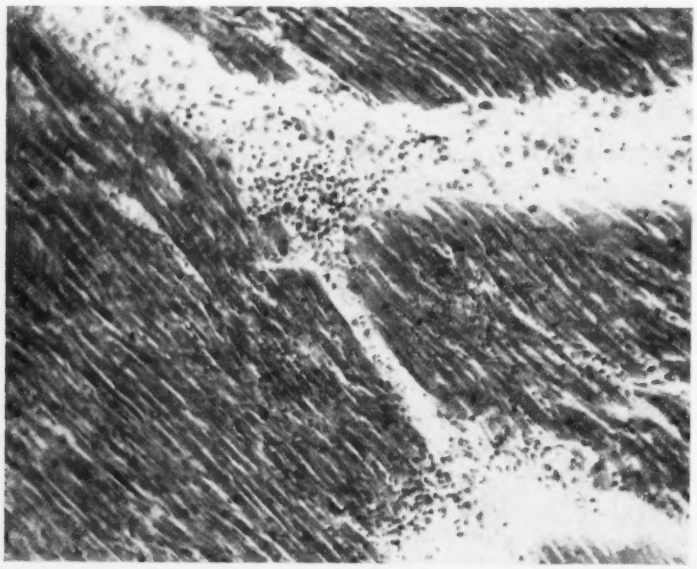


Fig. 2.—Mononuclear cell infiltration in an edematous connective tissue septum in the myocardium.

endothelium. Occasionally the entire vessel wall was loosely infiltrated with mononuclear cells.

The larger connective tissue septa were edematous and infiltrated to a varying degree with mononuclear cells (Fig. 2).

The more diffuse type of myocarditis was characterized by the presence of columns of mononuclear cells, chiefly plasma cells, lying in the connective tissue interstices between individual muscle fibers and in close relationship to capillaries (Figs. 3 and 4). The capillary endothelium often showed swelling and proliferation. Areas of focal hemorrhage were not unusual where this inflammatory reaction was severe. Degenerative changes in the cardiac muscle fibers varied from cloudy swelling, loss of striation, and fatty degeneration to actual necrosis. A resultant early fibroblastic proliferation was occasionally seen.

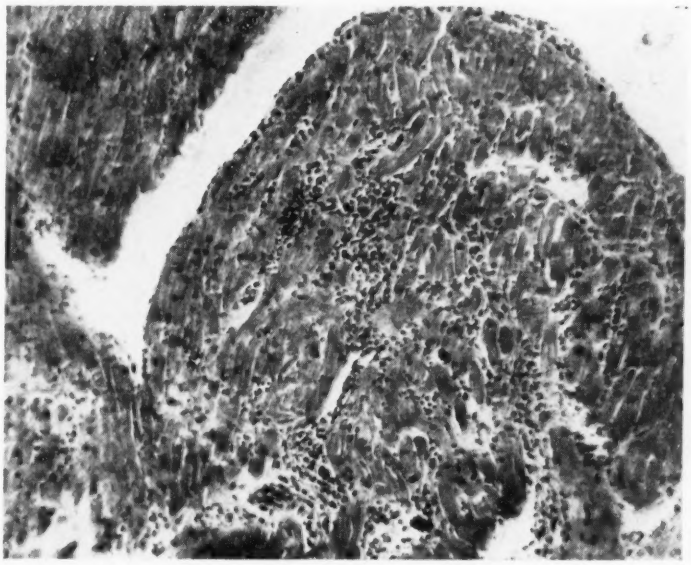


Fig. 3.—Diffuse myocarditis in tsutsugamushi disease.

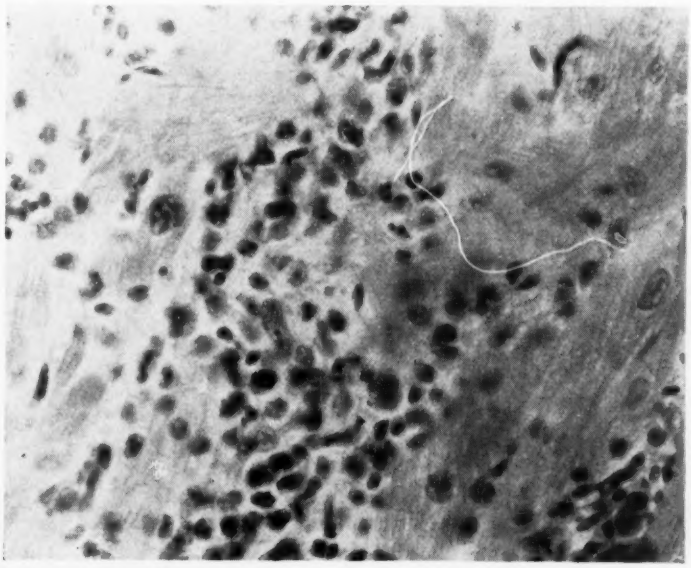


Fig. 4.—Diffuse myocarditis. High power to show nature of the cellular infiltration.

The *endocardium* was mildly edematous and the connective tissue layer slightly infiltrated with mononuclear cells. Occasionally, microscopic hemorrhages were present.

The *epicardium* usually exhibited increased vascularity. In all vessels changes were identical with those described in the myocardium. Focal hemorrhages were common. The tissue was sparsely infiltrated with mononuclear cells and occasional polymorphonuclear leucocytes. The serous endothelium was often swollen and proliferating. In several cases inflammatory changes had taken place in the subendothelial regions, and even in the media of the coronary arteries (Fig. 5).

The *aorta,* throughout its course, exhibited a mild acute aortitis characterized by perivascular infiltration of mononuclear cells about the vasa vasorum.

Fig. 5.—Coronary artery showing endangiitis and involvement of the media.

Fig. 6.—Aorta. Note the perivascular infiltration and the focal necrosis in the outer portion of the media.

Occasionally the vascular damage was severe and in two cases was attended by focal necrosis in the media with interruption of the elastic fibers in its outer portion (Fig. 6). Mild infiltration of mononuclear cells, plasma cells, lymphocytes, and histiocytes was occasionally seen in the subendothelial connective tissue of the intima.

The *lungs*, in all cases not obscured by complicating secondary pneumonia, showed an interstitial inflammatory reaction of such constant and typical features as to suggest a rickettsial origin. The arterioles and venules and sometimes the larger vessels of the interlobular septa, usually presented changes of varying degree such as perivascular infiltration of mononuclear cells, endothelial swelling and proliferation, infiltration of the wall, and not infrequently

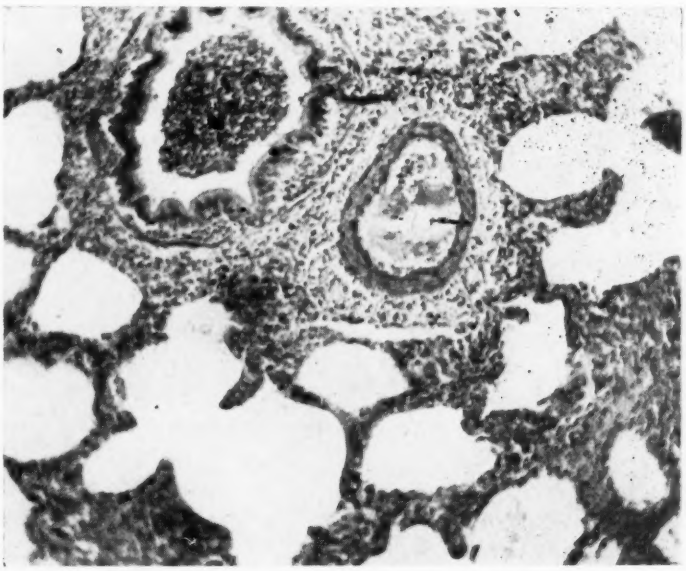


Fig. 7.—Interstitial pneumonitis in tsutsugamushi disease, probably of rickettsial origin. Note the inflammatory cells in the peribronchial regions and in the alveolar walls.

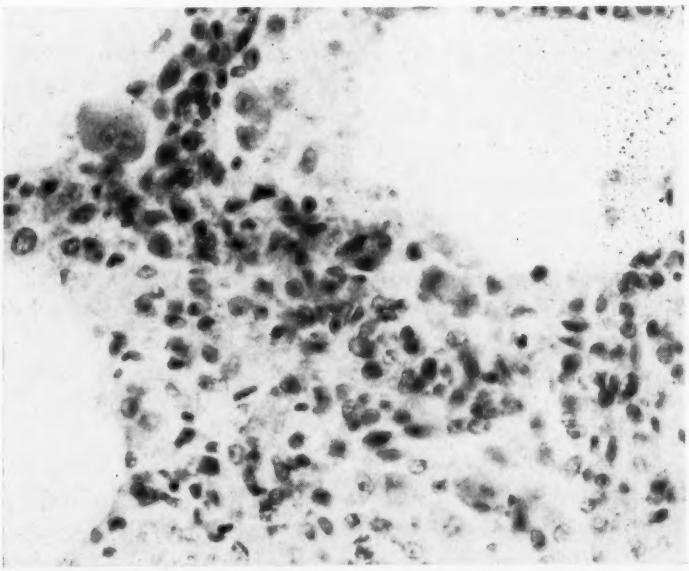


Fig. 8.—Interstitial pneumonitis. High power to show the types of cells present in the alveolar walls.

thrombosis. The interlobular septa were markedly edematous and diffusely infiltrated, chiefly by plasma cells and large mononuclear cells. The alveolar walls were congested and correspondingly infiltrated with mononuclears. The alveolar lining cells were swollen and proliferating. Most air spaces contained pink-staining edema fluid, a few mononuclear cells, and only occasional polymorphonuclear leucocytes. The alveoli contained no purulent exudate; neither did the primary and secondary bronchioles. A hyaline membrane lined the alveolar walls in several cases. Many areas were flooded with frank hemorrhage. These lesions varied considerably in severity and, as in the myocardium, were patchy in distribution. The pulmonary lesions are shown in Figs. 7 and 8. In some cases the basic condition of the lungs was overshadowed by a secondary acute bronchopneumonia and bronchiolitis which presented the usual histologic features.

The *pleura* showed changes similar to those in the epicardium, with edema, vascular and perivascular infiltration of mononuclear cells, endothelial swelling and proliferation, and occasionally thrombosis and hemorrhage. The connective tissue layer often was infiltrated with mononuclears; the serous cells commonly were swollen and proliferating.

The lesions in the *spleen* were those of acute splenitis with marked congestion of the pulp, frequently accompanied by hemorrhage and occasionally by infarction. The red pulp was densely infiltrated with mononuclear cells, plasma cells, large mononuclears, and lymphocytes. In some instances polymorphonuclear neutrophiles and eosinophiles were present in considerable numbers. Phagocytosis of erythrocytes and cellular debris by reticuloendothelial elements were prominent. The vascular changes were similar to those described in other organs. The Malpighian corpuscles were inconspicuous but often showed central hyaline necrosis.

The splenic capsule was often mildly infiltrated with mononuclear cells and polymorphonuclear leucocytes; the serous cells were swollen and proliferating. At times an exudate of fibrin, desquamated serous cells, and mononuclear cells adhered to the surface.

The *lymph nodes* presented a histologic picture similar to that seen in the spleen, characterized by acute lymphadenitis with congestion and occasionally hemorrhage and focal necrosis. The lymph sinuses were stuffed with large pale mononuclear cells, plasma cells, lymphocytes, and polymorphonuclear leucocytes. The fixed reticuloendothelial cells were prominent and increased in number, often containing phagocytized debris (Fig. 9). Focal hyaline necrosis was present in some follicles. These changes were usually more pronounced in the lymph nodes near the primary ulcer. Lymph nodes draining secondarily infected ulcers showed the usual picture of acute suppurative lymphadenitis.

The *gastrointestinal tract* was without distinctive changes except for vascular lesions of the type found in other organs. Areas of hemorrhage into the mucosa were common. In the case with massive terminal hemorrhage into the gastrointestinal tract, vascular lesions were unusually severe and there were innumerable foci of hemorrhage from the mucosal capillaries into the mucosa.

The mucosa was often infiltrated with considerable numbers of large mononuclear cells.

The *liver* presented no constant histologic features. The hepatic cells showed varying but usually mild degrees of cloudy swelling and fatty degeneration. The Kupffer cells were swollen and prominent; the cytoplasm at times was vacuolated, at times containing engulfed erythrocytes. Numerous mononuclear cells, both plasma cells and large mononuclear cells, were scattered through

the sinusoids. The portal spaces were mildly edematous and infiltrated with plasma cells and large mononuclear cells, with fewer lymphocytes, neutrophiles, and eosinophiles. In a few cases there was marked hepatic degeneration with necrosis. The degenerative changes were most marked about the central portion of the lobule, with relatively mild associated inflammatory reaction.

The *pancreas* showed slight microscopic change limited to perivascular infiltration in the supporting connective tissue. In rare instances vasculitis and perivasculitis were more severe and accompanied by edema and mild diffuse infiltration of the loose connective tissue bands sufficient to constitute an acute mild interstitial pancreatitis.

In the *suprarenal glands* clumps of mononuclear cells, chiefly plasma cells and large mononuclear cells, infiltrated the cortex and medulla. The larger vessels in the connective tissue of the central portion of the medulla showed perivascular cellular infiltration and occasionally actual evidence of vascular injury such as endothelial swelling, proliferation, or thrombosis. These changes varied considerably in severity within a given organ as well as from case to case.

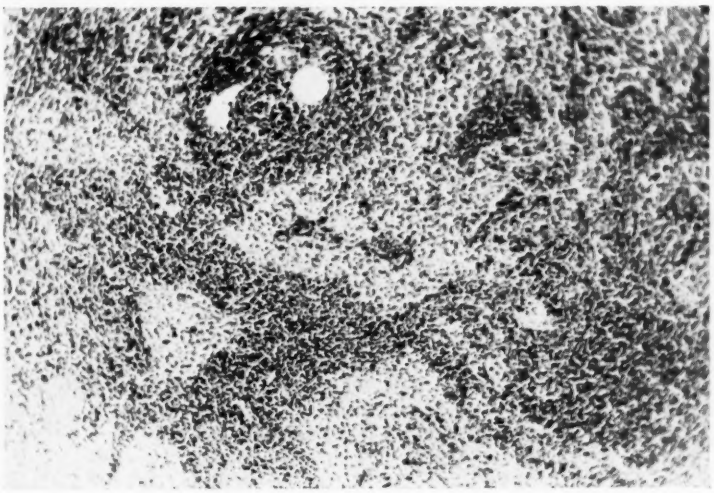


Fig. 9.—Mesenteric lymph node. Note the reticuloendothelial hyperplasia.

The *kidneys* showed characteristic focal interstitial lesions and, occasionally, evidence of severe vascular damage and glomerular injury. Usually, focal collections of plasma cells, large mononuclear cells, and lymphocytes, with occasional polymorphonuclear leucocytes, were scattered irregularly through the parenchyma, but they occurred most frequently about the junction of cortex and medulla. At times these collections encompassed two or three disintegrating tubular segments or a glomerulus. The long straight capillaries of the medulla were generally congested and frequently showed perivascular infiltration of mononuclear cells. The vessels throughout the kidney presented changes similar to those in other organs.

The epithelium of the convoluted tubules exhibited cloudy swelling. The tubules sometimes contained albuminous material.

The glomeruli were not uniformly affected; most of them were normally cellular and had well-filled capillaries; others, however, showed a definitely increased cellularity and were ischemic. Occasionally the capsular epithelium had proliferated and formed thin crescents. A few glomerular loops were

frankly necrotic. The glomerular spaces sometimes contained albuminous material, erythrocytes, or epithelial cells. At times pericapsular spaces were infiltrated by mononuclear cells.

Focal hemorrhage, occasionally seen in the parenchyma, was most common in the medulla. Recent anemic infarcts were seldom present in the cortex, but in these cases thrombi could sometimes be demonstrated in damaged arteries.

The *urinary bladder* showed only the vascular changes described, chiefly in the submucosa.

The *testicle* was not seriously affected and exhibited only characteristic vascular lesions, mild interstitial edema, and occasionally thrombosis and hemorrhage. The vascular lesions were much milder than those occurring in the heart, lung, and brain, and no more severe than those seen elsewhere.

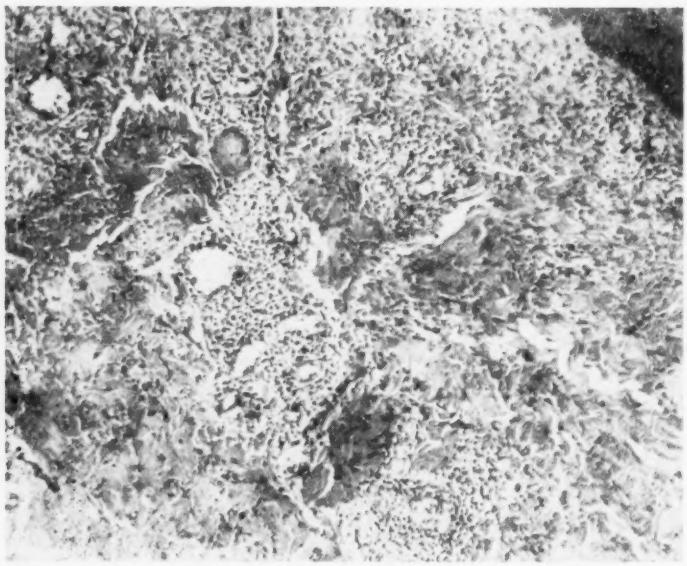


Fig. 10.—Subepithelial tissue in the primary cutaneous ulcer. Note the vasculitis and perivascular collections of mononuclear cells.

The *primary cutaneous ulcer* in the earlier phases showed coagulation necrosis of the epidermis and superficial layers of the corium. The characteristic vascular lesions were present in the corium (Fig. 10). Later ulceration occurred and the ulcer base was formed by degenerating leucocytes and cellular debris. The underlying corium was edematous. There was moderate diffuse cellular infiltration, chiefly lymphocytes, especially about the epidermal appendages as well as about nerve bundles; the appearance was that of a localized cellulitis. The inflammatory reaction did not tend to spread laterally but sometimes extended downward into the panniculus. Healing occurred with epithelization of the surface and absorption of the exudate. Vascular and perivascular infiltration, however, persisted even after surface healing had taken place.

Skin from areas which exhibited rash at the time of death showed comparatively mild changes. The capillary loops were moderately congested and surrounded by a few infiltrating cells, chiefly lymphocytes. The superficial vessels of the corium were similarly affected, but the deep vessels showed little change. Thrombosis and hemorrhage were exceedingly rare. The basal layer of the epidermis was mildly edematous.

The *bone marrow* sometimes was rich in plasma cells and large mononuclear cells.

The *brain* showed considerable variation from case to case. In some cases there was only mild perivascular infiltration of plasma cells and large mononuclear cells about the vessels of the leptomeninges with a few cells scattered through the arachnoid membrane. The arterioles, venules, and capillaries were congested to varying degrees.

In other cases a well-defined acute serous meningitis and an acute focal encephalitis were present. There was frequently marked perivascular infiltration of the vessels of the leptomeninges, and the arachnoid membrane and space were densely infiltrated with large pale mononuclear cells and plasma cells (Fig. 11). The vessels in all parts of the brain showed a similar cellular infiltration into the perivascular sheath; at times dense cuffs were formed. The vessels themselves exhibited the characteristic lesions described. Focal nodules

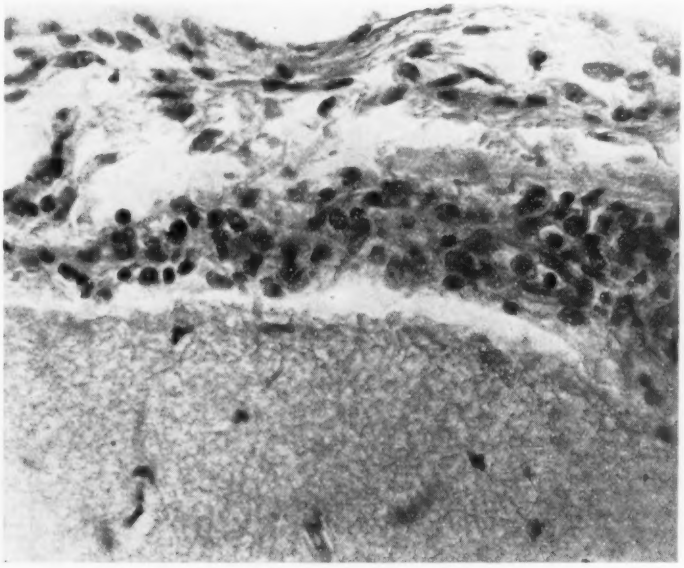


Fig. 11.—Mononuclear cell infiltration in the arachnoid membrane, giving the picture of mild meningitis.

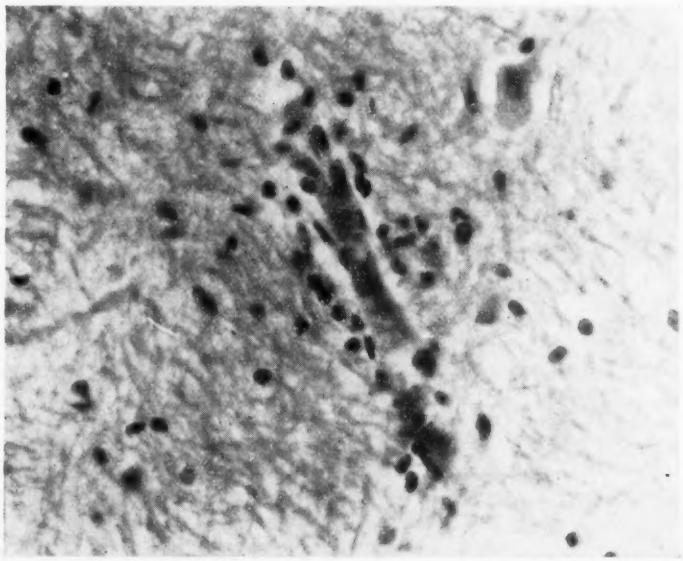


Fig. 12.—A damaged cerebral capillary, with early endothelial proliferation and perivascular reaction.

similar to those seen in typhus[13] and spotted fever[14] were scattered through the brain stem, cerebellum, and cerebrum. These had formed about small vessels or capillaries, probably incidental to vascular damage by rickettsial invasion in the endothelium. The nodules consisted of a loose conglomeration of irregularly arranged glial cells and large mononuclear cells, with occasional polymorphonuclear leucocytes. The surrounding brain tissue was edematous and lesions occasionally encompassed a neuron which showed varying degrees of chromatolysis. These lesions were seen in all stages of formation, from the early vascular type with marked perivascular cellular infiltration and endothelial proliferation, followed by thrombosis or closure of the lumen by endothelial proliferation and swelling, to the final, small, focal, granulomatous nodule (Figs. 12, 13, and 14); serial sections always proved the vascular relation.

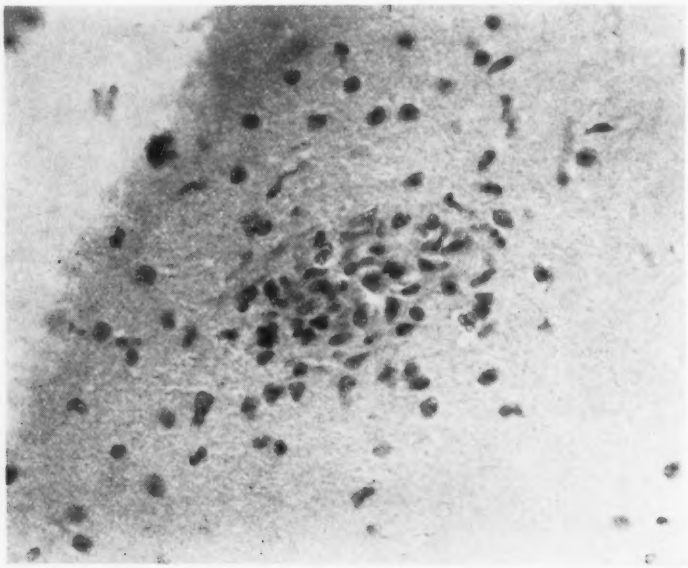


Fig. 13.—Early focal lesion in the cerebellum. Note the presence of several neutrophiles.

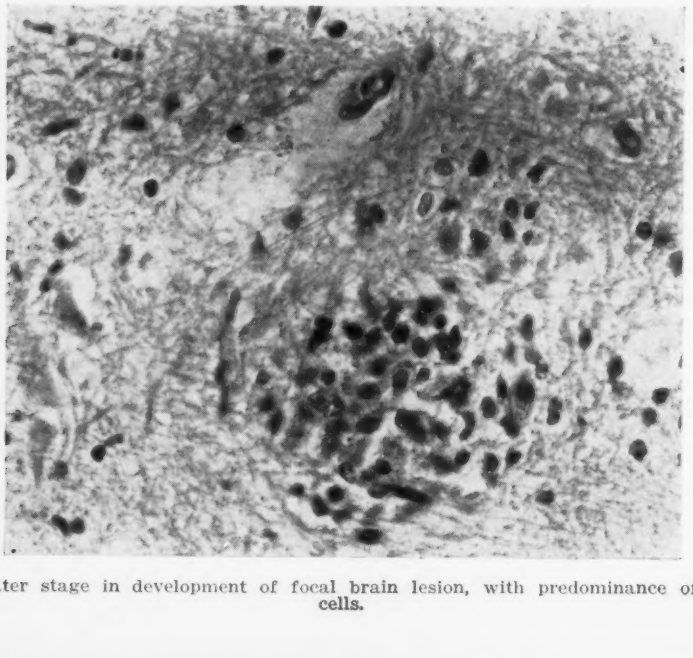


Fig. 14.—Later stage in development of focal brain lesion, with predominance of neuroglial cells.

Nodules were most frequent in the pons and medulla, less frequent in the basal ganglia, cerebellum, and cerebrum and spinal cord. It was unusual to see more than from one to three to a section. Punctate focal hemorrhages were often seen, scattered through brain stem and cortex. These hemorrhages sometimes were around nodules but often were unrelated to them. Nodules were seen much more frequently if death occurred after the twelfth day. In one case in which death occurred on the thirty-third day from pulmonary complications after an intervening afebrile period, the healing stage of the nodule was studied. The nodules were more compact than at earlier stages; plasma cells and large mononuclear cells were inconspicuous; cells with elongated oval nuclei and small glial elements predominated. The inflammatory reaction in the meninges and in vessels of the brain had practically disappeared.

Focal areas of demyelinization, which have been considered characteristic of spotted fever,[14] were rarely, if ever, encountered.

PATHOGENICITY AND PATHOLOGY IN EXPERIMENTAL ANIMALS

During the course of this study, the opportunity arose to observe the pathogenicity and pathology of tsutsugamushi disease in experimental animals, coincident with transmission studies carried out by other members of the Department of Bacteriology of the Third Medical Laboratory and members of the United States of America Typhus Commission.

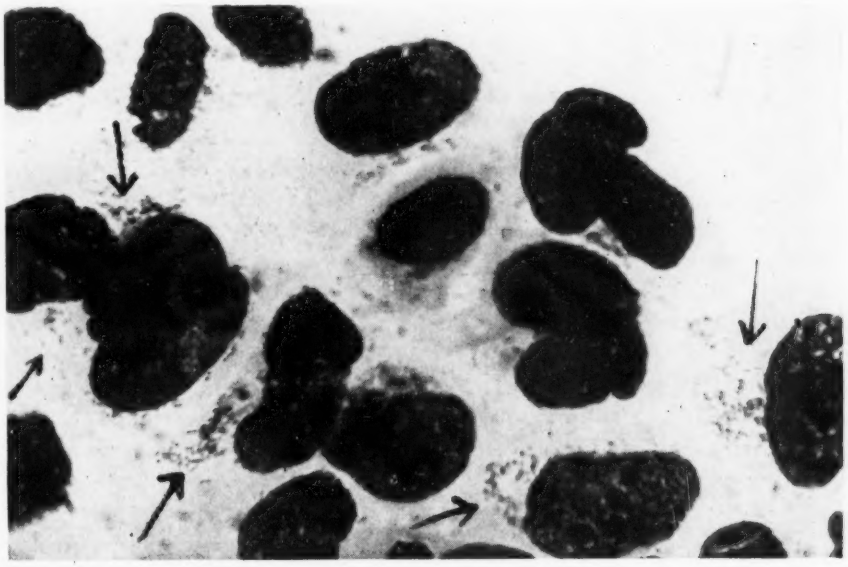


Fig. 15.—Serosal cells from peritoneal cavity of white mouse reacting to tsutsugamushi disease. Arrows point to loose collections of rickettsiae.

The White Mouse.—The white mouse seems to be the most satisfactory animal for initial isolation of tsutsugamushi disease from human patients. In thirteen out of fourteen cases, strains were isolated by the intraperitoneal injection of heart blood, obtained post mortem, into white mice. In the single unsuccessful experiment the sample of blood had remained in the refrigerator overnight. The patients from whom successful isolations were obtained died between the seventh and twentieth days of illness. The blood, if uncoagulated, may be injected directly or, if coagulated, may be ground and mixed with sterile saline solution. In either case, 0.3 c.c. was used for the injection.

In three out of three attempts, strains of tsutsugamushi disease were established in mice by the intraperitoneal injection of 0.3 c.c. of pericardial fluid. Pleural fluid likewise gave positive results in each of two trials, and peritoneal fluid gave a positive result in the single instance in which it was tested. Cerebrospinal fluid from the lateral ventricles gave negative results in two cases. Kouwenaar, however, isolated strains from the spinal fluid of his cases.[7]

Successfully infected mice appeared ill toward the end of the second week after injection and usually died before the fifteenth day. Autopsy revealed characteristic and constant changes. The peritoneal cavity was filled with cloudy mucinous fluid. The peritoneal surfaces showed marked capillary injection. The spleen was enlarged, mottled, and friable. The pleural cavities sometimes contained small amounts of cloudy fluid. The lungs were frequently congested and hemorrhagic. Giemsa-stained smears from the peritoneal exudate, spleen surface, or pleural fluid contained many large serous cells, with fewer large mononuclears and lymphocytes, and only occasional polymorphonuclear leucocytes. Typical intracellular rickettsiae were seen within the cytoplasm of the large serous cells (Fig. 15).

The Guinea Pig.—Guinea pigs may be infected either by intraperitoneal or intradermal inoculation and the site of infection results in slight differences in the pathologic pictures.

Infected by *intraperitoneal* inoculation with either human or wild rat strains, animals exhibit clinical evidence of illness during the second week—apathy, weakness, and dyspnea—and may die about the fifteenth day.

The gross pathologic findings are constant and characteristic. The peritoneal cavity contains upward of 10 c.c. of cloudy mucinous fluid. The peritoneal surfaces are lusterless, the capillaries injected. The spleen is enlarged and friable; its surface is lusterless and covered with delicate fibrinous exudate. The liver is enlarged and covered with a fibrinous exudate. Its external surfaces are mottled and reddish yellow and it is also friable. The kidneys and adrenals are grossly normal. There is no evidence of external scrotal swelling. The tunica vaginalis is usually without gross evidence of inflammation, although in some instances it may be slightly lusterless, its vessels injected, and occasionally punctate hemorrhage may be seen in the polar fat. The testicles appear grossly normal. The inguinal lymph nodes may be slightly enlarged and grayish pink. The pleural surfaces are smooth and glistening, and occasionally a small amount of cloudy pleural fluid may be present. The lungs show varying degrees of congestion and sometimes are hemorrhagic. The heart and brain are grossly normal.

Smears of peritoneal exudate and those from the spleen surface are rich in large serous cells, many of which contain intracellular rickettsiae within their cytoplasm.

The histopathologic findings include: (1) acute serous peritonitis with serous exudate; (2) acute splenitis with congestion and acute fibrinous perisplenitis; (3) mild acute inflammation of the tunica vaginalis with acute vasculitis and perivasculitis of the vessels of the tunica and polar fat, leucocytic infiltration, and fibrinous exudate; (4) acute fibrinous perihepatitis and acute hepatic degeneration with cloudy swelling, fatty degeneration, and focal necrosis; (5) acute renal congestion and cloudy swelling of the renal tubular epithelium; (6) varying degrees of acute pulmonary congestion, occasionally with hemorrhage; (7) mild acute focal and diffuse myocarditis, similar to lesions in the human being; (8) no typical typhus nodules in the brain, but usually marked congestion with mild perivascular round cell infiltration.

Animals inoculated *intradermally* on the anterior abdominal wall develop a primary ulcer with clinical evidence of illness during the second week—apathy, weakness, and dyspnea. In some animals death may occur about the fifteenth day.

The gross pathologic findings include a crusted, slightly red, cutaneous ulcer on the anterior abdominal wall at the site of inoculation. The peritoneal surfaces are covered with a thin layer of mucinous exudate, but little if any free fluid is present. The spleen is enlarged, friable, and deep purplish red. Its capsular surface is lusterless but without actual exudate. The liver is enlarged and friable; its capsular and cut surfaces are a mottled reddish yellow. The kidneys, pancreas, and adrenals are normal. There is no evidence of external scrotal swelling. The tunica vaginalis is pale and glistening. The testicles are grossly normal.

The pleural cavities may contain small amounts of cloudy fluid, and the pleural surfaces may be covered with a sticky exudate. The lungs are usually congested with dark hemorrhagic areas throughout. The heart and brain are grossly normal. Smears of peritoneal exudate and those from the surface of the spleen show numerous large serous cells, the cytoplasm of which contain many intracellular rickettsiae.

The histologic findings include: (1) acute primary cutaneous ulcer; (2) mild acute serous peritonitis; (3) acute splenitis with congestion; (4) severe acute focal encephalitis with multiple characteristic nodules in cerebrum and pons; (5) severe acute pulmonary congestion; (6) acute hepatic degeneration with cloudy swelling, severe fatty degeneration, and focal necrosis; (7) cloudy swelling of renal tubular epithelium.

Notably absent, in contrast to the intraperitoneally infected group, is the acute fibrinous perisplenitis, the acute fibrinous perihepatitis, and the inflammation and exudation in the tunica vaginalis. This last variation indicates that the inflammatory reaction in the tunica is a result of direct extension from the peritoneal cavity. However, intradermal inoculation leads to involvement of the central nervous system and the formation of typical nodules, while intraperitoneal inoculation does not. These results are similar to those obtained in murine typhus where the scrotal reaction appears to suppress the brain lesions.

The White Rat.—Fifteen rats injected intraperitoneally with a human strain of tsutsugamushi disease were killed and autopsied at intervals varying from the fourth to thirty-fourth day after injection. No gross or histopathologic findings could be attributed directly to the rickettsial infection in any case. There was no significant splenic enlargement as compared with control animals, and in no instance was there gross or microscopic evidence of perisplenic exudate. There was no evidence in any case of external scrotal swelling, and the tunica vaginalis was uniformly pale. Microscopically there was no evidence of leucocytic infiltration in the tunica. No nodules were demonstrable in the brain. Smears from peritoneal scraping and surface of the spleen were uniformly negative.

In spite of complete lack of any illness clinically or any positive pathologic findings, the infection was shown by Kohls, Armbrust, Irons, and Philip to be persistent in the brain of each animal.[15]

Bandicoots.—Seven apparently healthy wild bandicoots injected with a human strain of tsutsugamushi disease showed no clinical evidence of illness after from twenty-one to twenty-eight days. The animals were sacrificed and

thorough autopsy study revealed no lesions attributable to rickettsial infection. The spleens were not enlarged; there was no peritoneal exudate. Smears from peritoneal scrapings and spleen surface were negative. Rickettsia could not be recovered from spleen or brain in transmission studies by Kohls, Armbrust, Irons, and Philip.[15] Two uninfected animals killed in the bush were found to be identical both on gross and microscopic examination with the inoculated bandicoots.

The Wild Rat.—Six wild rats were caught on a small island very heavily infested with rats, where many cases of tsutsugamushi disease had occurred. These rats appeared in poor health generally, as evidenced by rough thin coats, malnutrition, and sluggishness. However, there was gross or histopathologic evidence of disease due to rickettsial infection in only one instance. In this case the tunica vaginalis presented mild vasculitis and perivasculitis with moderate perivascular infiltration of mononuclear cells, and an exudate of large mononuclears, polymorphonuclear leucocytes, and fibrin over the tunica at one point. No rickettsiae could be demonstrated in tissue sections. There was no peritoneal exudate or splenic enlargement, and focal brain lesions were not found. (A strain of rickettsia was recovered by Kohls, Armbrust, Irons, and Philip[15] from one of several other similar rats caught at random in the same place, but no attempt was made to recover the organisms from the animals examined.)

COMPARISON WITH EPIDEMIC TYPHUS AND SPOTTED FEVER

Epidemic typhus and spotted fever are remarkably similar to one another histopathologically, the most striking differences being the greater tendency to thrombosis and hemorrhage in spotted fever. The presence of areas of demyelinization in the brain in spotted fever[14] is a reflection of this greater tendency to thrombosis. In general, the tendency to thrombosis and hemorrhage appear to a smaller degree in tsutsugamushi disease than in either typhus or spotted fever.

The *local cutaneous ulcer* usual in tsutsugamushi disease is not seen in typhus. A similar lesion, however, occurs in fièvre boutonneuse, which is a strain of spotted fever.[17] It is noteworthy that the local lesion in the spotted fever group should be associated only with a strain of low mortality, while in the tsutsugamushi group it most often accompanies the highly virulent and fatal strains.

The tendency for the skin rash to become hemorrhagic is most pronounced in spotted fever and least pronounced in tsutsugamushi disease. These differences may be attributed to the more frequent occurrence of vascular thrombosis in the cutaneous vessels in cases of spotted fever and its relative rarity in tsutsugamushi disease; epidemic typhus occupies an intermediate position in this respect.

Involvement of serous membranes (pericardium, pleura, and peritoneum) is an outstanding feature of tsutsugamushi disease in man which is not seen in either epidemic typhus or spotted fever. In experimental animals, however, rickettsial peritonitis occurs in both typhus and spotted fever after intraperitoneal injection of rickettsiae but not after subcutaneous inoculation. In tsutsugamushi disease, the involvement of serous membranes is independent of the route of inoculation. In experimental typhus and spotted fever in the guinea pig, peritonitis is usually confined to the scrotal sac, where a lower temperature obtains. In starving guinea pigs, however, generalized peritonitis, similar to that seen in tsutsugamushi disease, occurs with regularity.[18]

The rickettsiae of tsutsugamushi disease apparently have a greater affinity for serosal cells than those of either typhus or spotted fever.

The myocarditis is tsutsugamushi disease appears to be identical qualitatively with that seen in typhus and in spotted fever, but from our observations we believe that it tends to be rather more severe in tsutsugamushi disease. Involvement of larger vessels, including the coronaries and the aorta, is perhaps somewhat commoner and more severe than in typhus or spotted fever.

The almost constant presence of interstitial pneumonia, which, if we are correct in our interpretation, is of rickettsial origin, has not been described for epidemic typhus. Wolbach and co-workers, for example, described the lungs in regions unaffected by bacterial pneumonia as essentially normal.[13] It appears certain, however, that interstitial pneumonitis may occur in epidemic typhus and in spotted fever, but the lesions in these diseases apparently are not as severe as in tsutsugamushi disease.

Generalized enlargement of lymph nodes, which was present in 67 per cent of our cases, also seems more common than in typhus or spotted fever.

The changes in the brain are similar in all essential features to those seen in typhus and spotted fever. The focal lesions appear identical. The absence of areas of demyelinization, such as have been described in spotted fever, is no doubt due to the infrequent occurrence of thrombotic occlusion in the cerebral vessels.

CLINICOPATHOLOGIC CORRELATION

All of the fifty-five patients in this series were treated in one or more evacuation or station hospitals in the forward or rear echelons of the combat zone. Overtaxed hospital services precluded the recording of any except the most essential clinical data or the performance of any except the most necessary laboratory examinations, so that the clinical information available was frequently meager. Nevertheless, an attempt has been made to correlate the clinical and pathologic features from the available data.

The pathologic findings in our series which may be regarded directly incident to the rickettsial disease are enumerated and amplified:

1. Generalized acute vasculitis and perivasculitis occurred to some degree in all patients. On a histologic basis the vasculitis could be classified as mild with perivascular infiltration alone in 37 per cent, moderately severe with perivascular infiltration, endothelial swelling, and proliferation in 45 per cent, and severe with extensive vascular damage, hemorrhage, and occasional thrombosis in 18 per cent. To vascular damage may be attributed the punctate hemorrhages so commonly found in the various organs. More rarely hemorrhages may be sufficiently severe to cause death, as in two patients of this series. Other results of vascular damage were the small infarcts occasionally observed in the lungs, kidneys, or spleen.

2. Acute focal or diffuse myocarditis was a feature in all cases and was classified histologically as mild, without myocardial necrosis in 47 per cent. Acute serous pericarditis with or without effusion was also commonly present. Acute aortitis of mild to moderate severity was usual.

3. Acute focal encephalitis with acute serous leptomeningitis, classified histologically as moderate, with only occasional nodules or punctate hemorrhage occurred in 37 per cent; severe with numerous brain lesions in 30 per cent. Minimal changes limited to mild perivascular infiltration, hardly justifying the diagnosis of encephalitis, occurred in 33 per cent.

4. Interstitial pneumonia, believed to be of rickettsial origin, with or without acute congestion and edema, was the only pulmonary lesion in 45 per cent. A similar lesion was probably present but complicated by secondary acute bronchopneumonia in 46 per cent, while in the remaining 9 per cent such a lesion, if present, was completely obscured by severe confluent secondary bronchopneumonia. Acute serous pleuritis with effusion was present in 53 per cent.

5. Acute splenitis with marked acute congestion and enlargement of the spleen was observed in 96 per cent of the series, often accompanied by mild acute perisplenitis.

6. Acute generalized lymphadenitis was present in 65 per cent, absent in 33 per cent, and not recorded in 2 per cent. Of those cases with acute lymphadenitis, three-fourths were classified histologically as mild; one-fourth, as moderately severe with hemorrhage, focal necrosis, and moderate lymphadenopathy.

7. Acute primary cutaneous ulcer with acute regional lymphadenitis was present in 89 per cent of our cases.

8. Acute hepatic degeneration with or without enlargement had taken place in 76 per cent. Of this group, approximately three-fourths showed only mild toxic degenerative changes with cloudy swelling and fatty degeneration, while the remainder exhibited severe hepatic degeneration with acute hepatitis and focal necrosis. Moderate jaundice was present clinically in two patients and minimal in two others.

9. Acute, focal, interstitial renal lesions were present in 76 per cent of the group; in one-half of these there was also moderate focal vascular and glomerular damage and inflammatory reaction. Cloudy swelling of the renal tubular epithelium was constant.

CLINICAL MANIFESTATIONS IN FATAL CASES

Prodromal symptoms develop after an eight- to ten-day incubation period and consist principally of malaise, headache, chills, and fever. Between the fourth and seventh days of the febrile period a macular rash appears, first on the face, later spreading to extremities and trunk. The axillary and inguinal lymph glands are frequently painful and tender. Cough, partial deafness, and generalized body hyperesthesia are usually present. The temperature is sustained and ranges from 103 to 105° F.

Several different chains of symptoms may appear as the disease progresses:

1. Circulatory failure, evidenced by increasing pulse rate and falling blood pressure, rapid shallow respirations, cyanosis, sweating, and cold clammy skin, generally appears in the second week. This syndrome, usually diagnosed clinically as peripheral vascular collapse, closely resembles that seen in surgical shock. Less frequently circulatory embarrassment occurs which is referable to acute myocarditis.

2. Widespread central nervous system involvement is manifested by twitchings, weakness of skeletal muscle groups, delirium, convulsions, and coma.

3. Pulmonary symptoms sometime predominate. In some cases clinical and roentgenographic examination is suggestive of virus pneumonia; in others sputum examination reveals the secondary bacterial nature of the disease.

4. Rarely hemorrhage may lead to a sudden fatal issue.

5. Clinical evidence of renal insufficiency is notably absent. However, subsequent to this study, one case was observed in which edema and gross hematuria were present.

6. In addition to the symptoms which can be primarily attributed to the rickettsial disease, some patients present disturbances referable to complicating or secondary diseases. This was particularly true in the group in which the course of the disease was prolonged. Bronchopneumonia was present in approximately one-half of the group. Acute malaria was a complication in eight of our patients and suppurative parotitis in one; and nonspecific diarrhea was frequent.

Death usually occurs toward the end of the second or in the early days of the third week. In our series, 95 per cent of the deaths occurred between the ninth and twentieth day of the disease, inclusive, the average being 14.6 days.

CAUSES OF DEATH

Clinically, death was ascribed to circulatory failure in about one-third of the patients, to respiratory failure in about one-third, and to cerebral involvement and miscellaneous complications in the remaining third.

1. Generalized acute vasculitis was a constant finding in our cases. Woodward and Bland[22] recently have emphasized the importance of this lesion as a cause of death in typhus fever. Our studies indicate that their conclusions may apply equally to tsutsugamushi disease. We regard as important the concept that patients with rickettsial vasculitis may die from peripheral circulatory collapse with its attendant physiologic disturbance just as surely as though they were suffering from surgical shock. Unfortunately, the clinical study of our cases did not distinguish sharply between peripheral circulatory collapse and myocardial failure. Woodward and Bland did not encounter a single instance of congestive heart failure in their series of thirty cases, two of which terminated fatally.

2. Myocarditis was a constant microscopic finding but was considered to be severe in only about half of our patients. In patients dying early (between the ninth and twelfth days of illness), severe myocarditis was somewhat more common than in those dying later.

The myocarditis is difficult to evaluate as a cause of death. We do not believe that it is of great importance when mild. In the more severe cases of tsutsugamushi disease, however, with degenerative changes in the myocardial fibers, death may be due to myocardial failure.

Six patients in this series were treated with sulfonamides, which probably have a detrimental effect in typhus[19] and spotted fever.[11]

As pointed out by Yeomans, Snyder, Murray, Zarafonetis, and Ecke,[20] penicillin is the bacteriostatic agent of choice in secondary bacterial infections complicating epidemic typhus and would probably be safer in tsutsugamushi disease. Sulfonamides have been shown to cause myocarditis, rather similar to rickettsial myocarditis, in sensitive individuals.[21] These drugs, however, were obviously not an important factor in the myocarditis described in our patients.

3. Encephalitis was present in variable degree in practically all of our patients. Severe encephalitis and involvement of the central nervous system were definitely more common in patients dying after the fourteenth day of illness. Cerebral involvement probably is the most important cause of death in about one-third of all cases, particularly if the patient remains in stupor or coma for several days before death.

4. Renal lesions are common, but at present we cannot evaluate them as a cause of death. Correlation of the lesions with renal function is difficult, because only rarely were urinalyses or functional tests recorded. However, it may be said that of the eight patients with mild focal interstitial lesions in whom

urinalyses were reported, five showed albumin 2 to 4 plus, with hyaline and granular casts and pus cells, while of the other three patients, urinalysis on admission was negative but the test was not repeated. Of the four patients with acute glomerulitis in whom urinalysis was recorded, albumin 2 plus, with numerous granular casts and erythrocytes, was present in three, while in the fourth, urinalysis was negative on admission and not repeated.

Blood chemistry was recorded in but one patient and the blood urea nitrogen was elevated to 59 mg. per cent; the typical renal lesions were mild without severe glomerular damage. In a fatal case not of this series, nitrogen retention was marked; the inflammatory reaction was severe.

Yeomans, Snyder, Murray, Zarafonetis, and Ecke[20] observed that epidemic typhus frequently produced severe impairment of renal function.

5. The interstitial pneumonitis which we regard as of rickettsial origin is believed to occur almost constantly in tsutsugamushi disease. This condition probably paves the way for bacterial pneumonia, but whether it is ever alone the cause of death is problematic. However, in one case seen following this study, the clinical picture was that of pneumonia. The lung changes were very severe and entirely of the interstitial type, and it was thought that death was attributable to the primary pneumonia. It is of interest of note that clinical and roentgenologic evidence of "atypical" or "virus" pneumonia is mentioned frequently as a part of the picture of tsutsugamushi disease.

Secondary bronchopneumonia accounted for death in about 20 per cent of our cases, most frequently when death occurred between the ninth and twelfth days of illness.

6. In discussing the cause of death, mention should be made of the possible importance of toxins set free from rickettsiae and circulating in the blood. Although this factor has not been carefully studied in tsutsugamushi disease, it is probably important. It may account for the diffuse myocarditis, but probably its greatest importance lies in the effect of the toxins on the peripheral capillaries. It may play an important part, perhaps even a more important part than the rickettsial vasculitis, in bringing about peripheral circulatory collapse.

SUMMARY AND CONCLUSIONS

A description of the lesions of tsutsugamushi disease in man, based on the study of fifty-five fatal cases, is presented. In general, the picture is that of acute generalized endangiitis, vasculitis, and perivasculitis, the lesions resembling those seen in epidemic typhus and spotted fever but with the following differences: (1) A primary local ulcer is almost constantly present; (2) local lymphadenitis is almost constant and generalized lymphadenitis is common; (3) vascular thrombosis is less conspicuous than in typhus and much less conspicuous than in spotted fever; (4) the cutaneous eruption very rarely becomes hemorrhagic; (5) rickettsial pericarditis, pleuritis, and peritonitis are common, while these lesions are not seen in typhus or spotted fever; (6) interstitial pneumonitis, believed to be of rickettsial origin, is almost constantly present; (7) inflammatory lesions of the larger arteries, notably the aorta, are apparently more conspicuous than in typhus or spotted fever.

The strains of tsutsugamushi disease studied were readily transmitted to the white mouse and to the guinea pig by the intraperitoneal injection of blood, pericardial fluid, pleural fluid, or peritoneal fluid. A conspicuous feature of the disease in experimental animals, regardless of the route of inoculation, is the occurrence of mucinous peritonitis with many serosal cells containing rickettsiae. Such a lesion does not occur in typhus or spotted fever except in

starving animals. The pathologic picture in the guinea pig resembles that in man. A serotal reaction does not occur. Focal brain lesions were found only after subcutaneous inoculation.

Morphologically, *R. tsutsugamushi* resembles the rickettsiae of typhus and spotted fever, with minor differences which are perhaps not constant. *R. tsutsugamushi* does not distend cells as does *R. prowazeki*, but the organisms tend to form loose intracellular groups of from ten to sixty organisms.

The cause of death in tsutsugamushi disease is discussed in the light of the lesions. It is believed that peripheral circulatory collapse, emphasized as a cause of death in epidemic typhus by Woodward and Bland, is likewise of outstanding importance in tsutsugamushi disease. The myocardial and cerebral lesions are also believed to be important causes of death. Secondary bronchopneumonia was the cause of death in about 20 per cent of our patients.

The authors are indebted to the many officers of the U. S. Army who submitted material for this study. Major C. A. Armbrust and Captain Edwin N. Irons carried out the bacteriologic procedures. Valuable technical assistance was rendered by Sgt. C. W. Strongman, Cpl. F. A. Preller, and T/5 A. M. Schultz. Pfc. C. G. Decker gave tireless clerical assistance.

REFERENCES

1. Lewthwaite, R., and Savoor, S. R.: Rickettsia Diseases of Malaya; Identity of Tsutsugamushi and Rural Typhus, Lancet **238**: 255, 305, 1940.
2. Ahlm, C. E., and Lipshutz, J.: Tsutsugamushi Fever in Southwest Pacific Theatre, J. A. M. A. **124**: 1095, 1944.
3. Farner, D. S., and Katsampes, C. P.: Tsutsugamushi Disease, U. S. Nav. M. Bull. **43**: 800, 1944.
4. Ishiwara, K., and Ogata, N.: Ein Neuer Beiträg zur Kenntnis der Eigenshaft des Tsutsugamushi-krankheit-virus, Tokyo Ijishinshi **2581**: 1555, 1936.
5. Nagayo, M., Miyagawa, Y., Mitamura, T., Tamiya, T., Sato, K., Hazato, H., and Imamura, A.: Ueber den Nachweis des Erregers der Tsutsugamushi-Krankheit, der Rickettsia orientalis, Jap. J. Exper. Med. **9**: 87, 1931.
6. Lewthwaite, R., and Savoor, S. R.: Typhus Group of Diseases in Malaya; Relation of Tsutsugamushi Disease (Including Rural Typhus) and Urban Typhus to Rocky Mountain Spotted Fever, With Special Reference to Cross-immunity Tests, Brit. J. Exper. Path. **17**: 461-472, 1936.
7. Kouwenaar, W.: Investigations on Rickettsial Diseases in Sumatra; Pathologic Anatomy of Human Mite Fever, Geneesk. tijdschr. v. Nederl. Indië. **80**: 1119-40, 1940.
8. Lewthwaite, R.: Pathology of Tropical Typhus (Rural Type) of Federated Malay States, J. Path. & Bact. **42**: 23, 1936.
9. Corbett, A. M.: Scrub Typhus, Bull. U. S. Army M. Dept. (No. 70), pp. 34-54, 1943.
10. Yoshida, S.: On Tissue Culture of Tsutsugamushi Virus (Rickettsia Tsutsugamushi), Kitasato Arch. Exper. Med. **12**: 324-337, 1935.
11. Pinkerton, H.: The Pathogenic Rickettsiae With Particular Reference to Their Nature, Biologic Properties, and Classification, Bact. Rev. **6**: 37, 1942.
12. Alexander, R. A., and Mason, J. H.: Studies of the Rickettsiae of the Typhus-Rocky Mountain Spotted Fever Group in South Africa, Onderstepoort J. of Vet. Sc. and Animal Industry, Pretoria **13**: 19-74, 1939.
13. Wolbach, S. B., Todd, J. L., and Palfrey, F. W.: The Etiology and Pathology of Typhus, Cambridge, Mass., 1922, Harvard University Press.
14. Lillie, R. D.: Pathology of Rocky Mountain Spotted Fever, Natl. Inst. Health Bull. No. 177, U. S. Public Health Service, 1941.
15. Personal communication; paper to be published.
16. Philip, C. B., and Kohls, G. M.: Personal communication; paper to be published.
17. Hass, G. M., and Pinkerton, H.: Spotted Fever. II. An Experimental Study of Fièvre Boutonneuse, J. Exper. Med. **64**: 601-623, 1936.
18. Pinkerton, H.: Unpublished observations.
19. Topping, N. H.: Experimental Rocky Mountain Spotted Fever and Endemic Typhus Treated With Prontosil or Sulfapyridine, Public Health Rep. **54**: 1143-1147, 1939.
20. Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S.: The Therapeutic Effect of Para-Aminobenzoicacid in Louse Borne Typhus Fever, J. A. M. A. **126**: 349, 1944.
21. French, C. E., and Weller, C. V.: Interstitial Myocarditis Following Clinical and Experimental Use of Sulfonamide Drugs, Am. J. Path. **18**: 109, 1942.
22. Woodward, T. E., and Bland, E. F.: Clinical Observations in Typhus Fever, With Special Reference to the Cardiovascular System, J. A. M. A. **126**: 287, 1944.
23. Moon, V. H.: Hemoconcentration as Related to Shock, Am. J. Clin. Path. **11**: 361, 1941.

CONGLUTINATION TEST FOR RH SENSITIZATION

ALEXANDER S. WIENER, A.B., M.D.
BROOKLYN, N. Y.

UNTIL recently, it was not clear why some individuals exhibited clinical evidence of marked sensitization to the Rh factor, yet the usual in vitro agglutination tests failed to reveal the presence in their sera of anti-Rh agglutinins. These puzzling cases have been explained, at least in part, by the discovery of the so-called Rh blocking antibodies,[1] which are characterized by their ability to combine specifically with Rh-positive cells but without producing a visible reaction. In fact, clinically, these blocking antibodies appear to be of greater significance than the Rh agglutinins. To detect their presence in cases where Rh agglutinins are not demonstrable, I have used the following technique: First, a drop (.05 c.c.) of a 2 per cent suspension (in terms of blood sediment) of Rh-positive red cells in saline solution and a drop of the patient's serum are mixed in a small test tube and allowed to react in a water bath at 38° C. for from thirty to sixty minutes. Then a drop of a suitable dilution of an active anti-Rh_0 agglutinating serum is added, and after an additional incubation period of from thirty to sixty minutes, the reactions are read. If blocking antibodies are present, no agglutination will occur, or the clumping will be markedly weakened.

In my hands[1] and in the hands of other investigators,[2, 3] the blocking test has proved a useful supplement to the usual agglutination test for Rh sensitivity. Puzzling intragroup hemolytic transfusion reactions, and instances of congenital hemolytic disease in which the maternal serum contains no demonstrable Rh agglutinins, have been clarified with the aid of the Rh blocking test. However, while the test is satisfactory as a diagnostic aid in cases of hemolytic disease, it has certain shortcomings when used as a compatibility test before blood transfusions. First, the blocking test takes twice as long as the ordinary tube agglutination tests; second, potent anti-Rh_0 serum is not always available. These objection have been met by Diamond and Abelson's simple slide test for Rh sensitization.[4] In this test, 0.2 c.c. of a concentrated (50 per cent) suspension of the test cells suspended in oxalated plasma or saline, preferably the former, is mixed with 0.1 c.c. of the patient's serum on an open slide. The test is attractive because of its simplicity, and the rapidity with which the reaction occurs is an advantage when the method is used as a compatibility test. The test is sensitive and, as Diamond and Abelson report, it gives positive results whenever either the tube agglutination or blocking tests are positive. These workers ascribed the success of their slide test to the use of heavy blood suspensions which, they stated, absorb the blocking antibodies, leaving the concealed agglutinins presumably present in the same serum free to act. Diamond and Abelson also state that the method is not suitable for determining the Rh blood types but offer no explanation for this. The slide test has the disadvantage that the mixtures dry rapidly because of the large surface, so the preparations are very perishable

From the Blood Transfusion Division of the Jewish Hospital of Brooklyn, and the Serological Laboratory of the Office of the Chief Medical Examiner of New York City.

The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Jewish Hospital of Brooklyn. The work was also aided in part by a grant from the United Hospital Fund of New York City.

Received for publication, May 19, 1945.

and the reactions must be read promptly. Moreover, the heavy blood suspensions and large surface on the slide favor the occurrence of troublesome rouleaux which at times are difficult to distinguish from true agglutination, while the thickness of the suspensions interferes with the microscopic reading, so that as a rule the readings have to be taken with the naked eye. The method also has the disadvantage that each blood serum must be tested separately, so that the technique is not as convenient as the tube technique for multiple testing. Accordingly, in the present paper I propose to describe a new tube test for Rh compatibility, and at the same time I shall offer an explanation for the reactions observed in Diamond and Abelson's slide test and Chown's[5] related capillary tube test.

The new technique of Rh testing described here differs from the standard method in the substitution of normal, compatible human plasma or serum in place of saline solution as a diluent in the tests. That is, all the blood suspensions are prepared in the usual manner and strength (2 per cent), except that serum or plasma is used as the diluent instead of saline. The serum being examined for Rh antibodies is tested undiluted or, if it is tested in dilutions as in titrations, compatible serum or plasma is used as the diluent instead of saline. For reasons which will soon become evident the name "conglutination test" is proposed for the new test, in order to distinguish it from the common agglutination test.

TABLE I. COMPARISON OF CONGLUTINATION, AGGLUTINATION, AND BLOCKING TESTS ON SERA FROM RH-NEGATIVE PATIENTS

NATURE OF TEST	TEMPERATURE OF TESTS	SERA FROM PATIENTS														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Conglutination*	Room	++	++	++	++	−	++	++	++	−	++	++	−	++	−	++±
Agglutination*	Room	−	−	−	−	−	−	++	−	−	−	++	−	+±	−	−
Conglutination	37.5° C.	++	++±	++	++	−	++±	++	++	−	++	++	−	++±	−	+++
Agglutination	37.5° C.	−	−	+	+±	−	+±	++	−	−	+	++	−	++±	−	−
Blocking†	37.5° C.	Bl.	Bl.	Bl.	0	0	Bl.	0	Bl.	0	Bl.	0	0	0	0	Bl.

*The conglutination and agglutination tests were set up in small test tubes by mixing a drop of a 2 per cent blood suspension (type Rh_0) with a drop of the serum being tested. In the agglutination tests, the blood cells were suspended in saline solution, while in the conglutination tests, the blood cells were suspended in plasma or serum.

†Bl., Blocking antibody present; 0, no blocking antibody detected.

In Table I is given a representative experiment in which the results of conglutination, agglutination, and blocking tests are compared on a series of blood sera from Rh-negative patients who were being tested for Rh sensitization. It will be seen that in every instance where agglutination was obtained in the usual tube tests, or where a positive blocking reaction occurred, strong clumping occurred in the conglutination test, while in no instance where the usual agglutination and blocking tests were both negative did clumping occur in the conglutination test.* The conglutination reaction appears to be far less sensitive to differences in temperature than is the usual agglutination reaction. Therefore, conglutination tests may be carried out at room temperature, which is an advantage if the conglutination test is used to determine compatibility preliminary to blood transfusion.

The name "conglutination test" was selected because of the resemblance of the reactions to the so-called conglutinin (co-agglutinin) phenomenon, which has been observed particularly in tests with bovine sera.[6-9] When red cells

*Subsequently, sera have been encountered which showed no Rh antibodies in the agglutination or blocking test, but in which Rh antibodies were demonstrated by the conglutination test.

(or bacteria) have been sensitized by their specific antibody and bovine serum is added to the combination, a pronounced cohesive and massy clumping of the sensitized cells occurs. Bovine serum alone has no effect unless the cells have been specifically sensitized. This phenomenon of "secondary molecular adhesion" is due to the presence in the bovine serum of a colloidal substance (possibly one or more of the serum proteins). The test described here occurs equally with fresh human serum or plasma, and with serum that has been heated at temperatures as high as 60° C. for thirty minutes, proving that the substance in question is not complement. On the other hand, even slight dilution of the serum or plasma with saline solution eliminates or weakens the reactions, probably because of the effect of the dilution on the colloidal properties of the serum. The substance apparently is adsorbed only after Rh-positive cells have been sensitized by the addition of either Rh agglutinins or Rh blocking antibodies and then brings about massive clumping of the red cells.

I believe that Diamond and Abelson's slide test and Chown's capillary tube test are both based on the same conglutinin phenomenon. Diamond and Abelson's hypothesis, that the excessive number of red cells used in the test absorbs the blocking antibody, leaving the agglutinins present in the same serum free to act, is contradicted by my observers because the reaction can also be brought about by sera containing blocking antibodies without agglutinins and in tests on diluted blood cell suspensions in serum or plasma. In their original report, Diamond and Abelson stated that the slide test could be carried out on 50 per cent cell suspensions of red cells in saline as well as plasma, but when I tried to duplicate the slide test using saline suspensions, I failed to get any convincing clumping.* The reason for this is now obvious, because the washed blood cells diluted in saline did not contain sufficient conglutinin. With regard to Diamond and Abelson's remark concerning the unsuitability of the slide test (and this would apply also to Chown's capillary tube test) for determining the Rh blood types, this does not hold when antisera containing the agglutinins anti-Rh_o, anti-Rh′, anti-Rh″, and anti-Hr separately are used in the tests. On the other hand, if the anti-Rh′ and anti-Rh″ reagents have been prepared from anti-Rh_o′ and anti-Rh_o″ sera by the addition of anti-Rh_o blocking serum,[10, 11] or if natural anti-Rh′ and anti-Rh″ sera containing Rh_0 blocking antibodies are used, Rh typing will not be possible because the blocking antibodies themselves will bring about the clumping under the conditions which exist in the slide test and capillary tube test.

Incidentally, all the blocking sera obtained by me to date (and also by Race and Taylor[2]) have had specificities corresponding to anti-Rh_o. Apparently, blocking sera of specificities corresponding to anti-Rh′, anti-Rh″, and anti-Hr, like those described by Diamond and Abelson,[3] must be extremely rare.

The clinical and theoretical implications and the practical applications of the new conglutination test for Rh sensitization are manifold. First of all, it supplies the missing link to the problem of the pathogenesis of congenital hemolytic disease (erythroblastosis fetalis). Up to now, the descriptions of the pathogenesis of congenital hemolytic disease have failed to take into account the colloidal state of the infant's or fetus' own blood serum, which presumably would be quite different from that of adults. (Investigations on conglutinin in fetal and infant's sera are now in progress.) The puzzling observation that infants apparently normal at birth may suddenly exhibit clinical signs of severe hemolysis with resulting death within several hours or days may be due

*Dr. John Elliott (personal communication) found that saline suspensions were agglutinated irregularly in the slide test. Red cells suspended in 25 per cent albumin reacted, while red cells suspended in globulin were unsuitable for the test.

to the effect of the conglutinin, which perhaps does not appear in the infant's serum until shortly before or after birth. Titration tests on maternal sera using the conglutinin technique have in a few cases revealed the presence of high-titered Rh antibodies where the usual tests showed the presence of only weak agglutinins or blocking antibodies of low titer. An important practical application is that the conglutination test, like Diamond and Abelson's slide test, makes available for use for Rh testing (determinations made with anti-Rh_0 antibodies only[13]) numerous sera which up to now have been discarded as unsuitable for use. By increasing the supply of anti-Rh serum, the conglutinin test should considerably increase the clinical usefulness of the Rh diagnostic tests; for example, for prenatal testing of pregnant women.

While the technique described deviates from that customarily used for Rh agglutination, actually it is simpler in certain respects. First, the reactions appear to be almost as strong at room temperature as at body temperature so that use of a water bath or incubator is unnecessary. Second, it is unnecessary to wash the cells; in fact, washing the cells is to be avoided. While it is desirable to obtain the blood to be tested by venipuncture, this is not essential if this is not feasible, because the red cells can be suspended in group AB serum (or plasma) stored in the refrigerator or any serum of a compatible group. Blocking serum can be used as an anti-Rh_0 agglutinating reagent like ordinary anti-Rh_0 serum, if the isoagglutinins they contain are neutralized by the addition of *dried* A and B substances,[14] and the sera are diluted to the desired titer with inactivated group AB serum instead of saline. Of course, the blood cells being tested must be suspended in serum instead of saline.

The observations reported may serve to reconcile the opposing views concerning the specific or nonspecific nature of the second stage of the agglutination and precipitation reactions.[15] With Herman, I have observed[16] that in mixed agglutination systems where diluted sera are used, the agglutination is specific so that each clump contains cells of the same kind only. In tests with undiluted sera, on the other hand, mixed clumps were obtained. Probably, in the latter instance we were dealing with the conglutinin phenomenon rather than with a typical agglutination reaction. It seems that in typical agglutination reactions the second stage as well as the first stage is specific, while in conglutination reactions the second stage is nonspecific. Similar effects probably hold in the case of precipitation reactions,[17] except that in the precipitation tests undiluted serum is used more often than in agglutination tests.

Experiments are in progress to apply the conglutination reaction to other antigen-antibody systems. For example, it may prove possible to improve the speed and intensity of the reactions of anti-A and anti-B grouping sera with the aid of this phenomenon. In fact, it seems likely that the increased avidity which is exhibited by the antisera prepared by globulin fractionation may be explained on this basis. As another example, the test may prove useful in explaining the pathogenesis of the so-called acquired hemolytic anemias.[18] Under certain conditions, a sudden breakdown of blood cells may occur; for example, due to infection with viruses having an affinity for erythrocytes, or the action of hemolytic poisons (sulfanilamide, fava bean, etc.), during the crisis of familial hemolytic icterus, from infectious agents (malarial parasites, streptococcus hemolyticus infections, etc.), trauma (hemorrhage into tissues with formation of large hematomas), etc. As a rule, after recovery from the acute insult no sequelae result, but in rare susceptible individuals, one could conceive that the elimination of the destroyed red cells may result in the production of autoantibodies. These autohemolysins could destroy more of the individual's red

cells, and a vicious cycle could result with the picture of a protracted hemolytic anemia sometimes continuing long after the initial noxious agent has ceased acting. One difficulty with this theory is that attempts to demonstrate auto-antibodies in the patient's serum have up to now yielded equivocal results in the great majority of cases. Thus, when autoagglutinins were found, these mostly were active only at low temperatures and so did not differ from the cold autoagglutinins occurring normally in the sera of most individuals, except for their titer. Recently, in a case of acquired hemolytic anemia, by using the conglutination technique, I succeeded in demonstrating autoagglutination in vitro at body temperature as well as in the refrigerator. If in future cases of acquired hemolytic anemia the conglutination test proves equally successful, this may help solve the problem of the pathogenesis of these obscure anemias.

COMMENT

It may be of interest to review how the test-tube conglutination test for Rh sensitization described here was developed. When the report of Diamond and Abelson appeared, describing their important slide test for Rh sensitization, naturally an attempt was made to reproduce their results in our laboratory. When 50 per cent blood suspensions in saline solution were used, one of the alternative methods mentioned in their article, the results were unconvincing. A visit was then paid to Dr. Diamond's laboratory at his invitation, where he gave a most impressive demonstration of the test. When again, upon returning to our laboratory, despite Dr. Diamond's demonstration and explanation of the slide test, we were still unable to duplicate his results, it was decided to investigate the mechanism of the reaction in an attempt to explain the discrepancy. It was then found that it was not the strength of the blood suspension but the avoidance of the introduction of saline solution into the mixture that spelled the difference between success and failure in the test.

For practical purposes, as in compatibility tests before blood transfusions, or wherever a rapid diagnosis is essential, the slide test of Diamond and Abelson will be the method of choice. The conglutination test herein described may prove most useful for more precise work, particularly in more exacting theoretical investigations.

While this paper was in press, an investigation was started to determine the results of slide tests and tube conglutination tests on the serum of infants with hemolytic disease. With the aid of these techniques, Rh antibodies have thus far been demonstrated without difficulty in three successive cases. Significantly, the blood cells of these infants were only weakly agglutinated or failed to agglutinate with standard anti-Rh_0 serum in agglutination tests carried out by the customary tube technique, presumably due to adsorption onto the surface of the infant's cells of Rh_0 blocking antibodies derived from the mother through the placenta. In support of this idea was the observation that in the slide test and/or tube conglutination test, autoagglutination of the infant's cells by its own serum was readily demonstrated. This also explains the occasional paradoxical reports submitted by laboratories unaware of this phenomenon that the infant as well as the mother is Rh negative (or belongs to type Rh′ or Rh″) even though the infant has hemolytic disease. Investigations are being conducted to determine how long the Rh antibodies persist in the infant's circulation. In one infant, treated by two transfusions of a total of 200 c.c. of Rh-negative blood, Rh antibodies were still demonstrable two weeks after birth in tests carried out with dilutions of the infant's serum as high as 1:5.

In the meantime, our attention has also been called to the important monograph of K. O. Pedersen (Ultracentrifugal Studies on Serum and Serum Fractions, Uppsala, Sweden, 1945), in which are described observations of significance in relation to the conglutination test. Pedersen discusses in detail a so-called X protein, which makes up from 20 to 50 per cent of the total serum proteins and is a protein of very high molecular weight consisting of a complex of albumin, globulin, and phospholipid. X protein dissociates readily

upon slight dilution with saline solution, like the colloidal substance responsible for the conglutination reaction, and observations on at least one individual suggest that it may be increased as a result of immunization. Moreover, in cow's fetus and newborn calves and foals, Pedersen has found a globulin of low molecular weight, named fetuin by him, which may be a precursor of one of the constituents of X protein. Fetuin is present only in small amounts in human umbilical cord blood, which is in line with the suggestion made in this paper that there may be a change in the colloidal properties of human serum toward the end of pregnancy or at about the time of birth.

SUMMARY

A new tube test (conglutination test) for Rh sensitization has been described. The technique entails the use of red cells suspended in compatible human plasma (or serum) instead of saline solution, while the serum being examined for its agglutinin content is tested either undiluted or diluted with plasma or serum. Dilution of the mixture with saline solution or any other crystalline solution is strictly avoided. Positive reactions are obtained in all cases where the serum being tested contains Rh agglutinins detectable for the usual tube technique and/or Rh blocking antibodies, so the results are similar to those obtained by the slide technique of Diamond and Abelson. The reaction seems to depend upon a thermostable colloidal substance in the serum (or plasma) which brings about massive agglutination of Rh-positive cells but only after they have been sensitized by their specific antibody.

The observations described supply one of the missing links in the theories of the pathogenesis of congenital hemolytic disease and intragroup hemolytic transfusion reactions. Moreover, like Diamond and Abelson's slide test, they help increase the supply of Rh testing serum available for clinical use by rendering useful sera that are of no value in tests performed by the customary techniques.

REFERENCES

1. Wiener, A. S.: A New Test (Blocking Test) for Rh Sensitization, Proc. Soc. Exper. Biol. & Med. **56**: 173, 1944.
2. Race, R. R., and Taylor, G. L.: The Incomplete Antibody in Rh Immunization, Brit. M. J. **2**: 756, 1944.
3. Diamond, L. K., and Abelson, N. M.: Importance of Rh Inhibitor Substance in Anti-Rh Serum, J. Clin. Investigation **24**: 122, 1945.
4. Diamond, L. K., and Abelson, N. M.: The Determination of Anti-Rh Agglutinins. An Accurate and Rapid Slide Test, J. Lab. & Clin. Med. **30**: 204, 1945.
5. Chown, B.: Rapid, Simple and Economic Method for Rh Agglutination, Am. J. Clin. Path. (Tech. Suppl.) **14**: No. 11, 1944.
6. Muir, R., and Browning, C. H.: On the Action of Complement as Agglutinin, J. Hyg. **6**: 20, 1906.
7. Bordet, J., and Streng, O.: Les Phénomènes d'Absorption et la Conglutinin du Serum de Boeuf, Centralbl. f. Bakt. **49**: 260, 1909.
8. Dean, H. R.: On Factors Concerned in Agglutination, Proc. Roy. Soc. B. **84**: 416, 1911-12.
9. Leschley, W. Z.: Versuche uber Konglutination, Ztschr. f. Immunitäts. **25**: 219, 1916.
10. Wiener, A. S.: The Rh Blood Types and Some of Their Applications, Am. J. Clin. Path. **15**: 106, 1945.
11. Wiener, A. S., Davidsohn, I., and Potter, E. L.: Heredity of the Rh Blood Types. II. Observations on the Relation of Factor Hr to the Rh Blood Types, J. Exper. Med. **81**: 63, 1945.
12. Race, R. R.: An 'Incomplete' Antibody in Human Serum, Nature **153**: 771, 1944.
13. Wiener, A. S.: The Rh Blood Factors, J. A. M. A. **127**: 294, 1945.
14. Wiener, A. S., and Forer, S.: A Human Serum Containing Four Distinct Iso-agglutinins, Proc. Soc. Exper. Biol. & Med. **47**: 215, 1941.
15. Boyd, W. C.: Fundamentals of Immunology, New York, 1943, Interscience Press, p. 236.
16. Wiener, A. S., and Herman, M.: Second Stage of the Agglutinative Reaction, J. Immunol. **36**: 255, 1939.
17. Heidelberger, M., and Kendall, F. E.: Precipitin Reaction Between Type III. Pneumococcus Polysaccharide and Homologous Antibody; Quantitative Study and Theory of Reaction Mechanism, J. Exper. Med. **61**: 563, 1945.
18. Wiener, A. S.: Blood Groups and Transfusion, ed. 3, Springfield, Ill., 1943, Charles C Thomas, p. 42.

THE DETECTION OF RH SENSITIZATION: EVALUATION OF TESTS FOR RH ANTIBODIES

LOUIS K. DIAMOND, M.D., BOSTON, MASS., AND NEVA M. ABELSON, M.D.
PHILADELPHIA, PA.

THE detection of Rh sensitization is a problem with which those who deal with blood transfusion or with obstetric patients are frequently faced. As we[1] have previously pointed out, the laboratory recognition of such sensitization may avert or resolve hemolytic transfusion reactions. All too frequently, patients are exposed to the discomforts and hazards of incompatible transfusion, or denied further transfusion, because a previous reaction has not been satisfactorily explained. A substantial number of these patients can be treated successfully if the clinician has recourse to adequate means of detecting the source of incompatibility. Furthermore, the employment of a reliable method of detecting Rh antibodies is an important aid in the management of pregnancies of Rh-negative women whose husbands are Rh positive. Many couples who have learned of the importance of the Rh factor, but who have acquired inadequate information or misconceptions concerning it, come to the physician with questions and with apprehensions with which he can deal honestly and capably only if he has at his disposal satisfactory methods for Rh typing and for determination of Rh antibodies. Trustworthy reports of the absence of antibodies are reassuring both to the physician and to the patient. Unfortunately, the idea has become prevalent that most of the complications of pregnancy (and particularly miscarriages and abortions) in Rh-negative women are due to Rh incompatibility. This misapprehension can best be corrected by the provision of accurate and convincing laboratory data. Moreover, the demonstration that antibodies are present serves as a guide for prognosis and permits mobilization of the safeguards now available for the mother and the erythroblastotic infant, thereby converting these deliveries from unpredictable emergencies into medical problems over which a certain amount of control can be exercised.

In the past, the laboratory confirmation of Rh sensitization has usually been dependent upon a single test which has proved inadequate in as many as from 40 to 50 per cent of instances. Laboratory means for detection of Rh antibodies have, therefore, gained a poor reputation. However, not a single, but several, methods of detection are now available, and it seems worth while to assess their value as laboratory aids to clinical medicine. These tests are conveniently discussed herewith.

THE BIOLOGIC TEST

The biologic test has been proposed and used by Wiener and co-workers.[2, 3] It involves the injection of a quantity (50 c.c.) of Rh-positive cells into the Rh-negative patient suspected of possessing abnormal antibodies, or the in-

From the Children's and Infants' Hospitals and the Department of Pediatrics, Harvard Medical School, Boston, Mass., the Department of Pediatrics, University of Pennsylvania Medical School, Philadelphia, and the Blood Grouping Laboratory, Boston, Mass.

A part of the work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard College.

Received for publication, June 14, 1945.

jection of this amount of blood from a proposed donor into a patient who has had a prevoius transfusion reaction. After an hour, the patient's plasma and serum are compared with plasma and serum drawn at the beginning of the test. If there is a change in the depth of the color of the plasma and serum (that is, an increase either in bilirubin or in free hemoglobin), the blood given is assumed to be incompatible. If there is no alteration, and an emergency exists, a full transfusion is given. If time permits, however, the test is repeated with a second 50 c.c. Care must be taken that factors tending to hemolyze the red cells are eliminated or avoided, and it is for this reason that specimens of both serum and plasma are withdrawn.

The test has obvious clinical disadvantages. Besides necessitating several venipunctures over a considerable period of time, it presents, first, the immediate danger of transfusion reaction. Highly sensitized patients (whose antibodies have in the past often been difficult to detect in vitro) may have a moderate reaction to as little as 5 or 10 c.c. of incompatible blood, and the reaction to as much as 50 or 100 c.c. may be one of serious proportions. In one case which we observed, chills, fever, and abdominal pain followed the intravenous injection of only 5 c.c. of packed red cells into a patient who had previously been delivered of a baby with erythroblastosis fetalis. Second, the injected red cells are potentially antigenic. There is no doubt that the infusion of 50 or 100 c.c. of Rh-positive blood into an Rh-negative person (and especially a woman in the childbearing age) may be a factor in initiating or restimulating antibody production. The use of the biologic test is, therefore, no longer justified except in the rare case where in vitro methods may yield equivocal results. As Weiner[3] has pointed out, this test was never meant to supplant, but rather to supplement, the in vitro detection of sensitization and was intended only for emergency situations when laboratory assistance is not readily available. It is especially no longer necessary since the open slide test and its test-tube modification (described later) are now available for dependable determination of Rh antibodies.

THE MODIFIED COMPATIBILITY, OR TUBE-INCUBATION, TEST

Levine[4] found that Rh agglutinins studied by him reacted more intensely at 37° C. than at 20° C. and suggested that incubation was essential for their detection.[5] He recommended that in performing compatibility tests in cases where isoimmunization might be a factor, the patient's serum and the prospective donor's cells be incubated for from fifteen to thirty minutes at 37° C. The method has been modified for detection of Rh antibodies as follows:

Two drops of a 2 per cent suspension of Group 0, Rh-positive red cells in normal saline (roughly one full drop of blood to about 1 c.c. of saline) are placed in a small test tube. Two drops of the unknown serum are added. After shaking, the tube is placed in a 37° C. water bath for one hour, then gently agitated and centrifuged at 500 revolutions for one minute. The character of the cell button is noted (a smooth outline is characteristic of the negative test). The tube is then gently tilted a half dozen times, to loosen some of the sediment from the bottom of the tube, and observed. Gross clumping can be seen with the naked eye or with the aid of a hand lens. If no gross clumping is observed, a drop of the suspension is examined microscopically for confirmation of absence of agglutination. The result is usually controlled by testing also with an Rh-negative cell, and the specificity of the serum is determined by testing with several different Rh-positive cells (some

belonging to the phenotype Rh_0' and some of the phenotype Rh_0''). By experienced technicians, sensitization may thus be detected in a substantial number of the cases. With inexperienced workers, this test is much less satisfactory, since in many cases the agglutinates are so fine as not to be apparent to the uncritical eye and so fragile as to be easily broken up by careless or vigorous handling. In an analysis of 500 cases of Rh sensitization (proved by hemolytic transfusion reaction or the delivery of an erythroblastotic infant), 265 sera (53 per cent) produced moderate or strong agglutination with the majority of Rh-positive cells. In addition, 57 sera (11.2 per cent) produced very weak agglutination, and 22 (4.4 per cent) produced agglutination only with cells of specificity Rh_0'.

This test, despite its limitations, has certain important advantages. If the physician has had the opportunity to follow the patient from the onset of sensitization, and particularly if quantitative estimations of the amount of antibody present in the blood stream are regularly made (that is, titration by the serial dilution method), it provides a useful guide to prognosis. Generally speaking, if only agglutinating antibodies are found by this method, and if these have begun to increase in strength only in the latter months of pregnancy, the fetus has a fair chance of survival. In these cases the mother may be spared further sensitization, and the infant further damage, by a reasonably early induction of labor. On the other hand, a single test showing the presence of agglutinins does not lend itself so easily to clinical interpretation, since a low titer of agglutinating antibodies (as determined by this method) may denote either early sensitization or profound late sensitization, depending upon the presence of inhibitor substances or "blocking" antibodies (described later and elsewhere). For this reason, sera of pregnant, Rh-negative patients should be tested at regular intervals.

THE "BLOCKING" TEST

The "blocking" test has recently been proposed by Wiener.[6] It is based upon a discovery made independently by Race[7] and by Wiener that many persons sensitized to the Rh factor develop antibodies which are capable of combining with the Rh-positive erythrocyte without a visible reaction, and which are, in fact, capable of preventing the usual visible reaction with potent, standard (Rh_0) sera. As originally described by Wiener, this test was performed by addition of the unknown serum to a 2 per cent suspension of Rh-positive cells, followed by incubation in the water bath at 38° C. for from thirty to sixty minutes. The tubes were then centrifuged and, in some cases, the supernatant fluid withdrawn. The results of the test were not significantly altered by this latter procedure. A drop of potent anti-Rh_0 serum was thereupon added and the mixture incubated for another thirty to sixty minutes, following which, after centrifugation, the tubes were observed for inhibition of the expected agglutination. We have found it possible to shorten the procedure and to perform the test in conjunction with the tube-incubation method for agglutininins. At the same time the latter test is set up, two additional suspensions of Rh-positive cells are prepared. The cells are so selected that one belongs to the phenotype Rh_0' and the other to the phenotype Rh_0''. The unknown serum (2 drops) and the potent agglutinating serum (1 drop of a dilution of titer 4 to 8 or whatever final dilution just yields grossly visible agglutination) are added at the same time. A parallel control is employed, using saline instead of the unknown serum specimen. The tubes are then incubated and centrifuged in the same manner as described, and search for

agglutination is carried out. If agglutination occurs in the control tube but is absent or inhibited in the tube containing the unknown serum, blocking antibodies may be assumed to be present. There are several points to which particular attention must be paid in the performance of this test:

1. The Rh-positive cells used should belong to the phenotypes Rh_1 (or Rh_0') and Rh_2 (or Rh_0'').

2. The specificity of the agglutinating serum should be carefully determined, because by far the greatest number of blocking antibodies are effective only against Rh_0 sera. An Rh_0' or Rh_0'' serum should not be used; otherwise the Rh′ and Rh″ agglutinins will interfere with the Rh_1 (or Rh_0') and Rh_2 (or Rh_0'') cells, respectively.

3. The test should not be set up without a control; otherwise partial inhibition of the expected agglutination may not be appreciated.

In our analysis (see Table I), 120 sera (24 per cent) produced strong inhibition, 57 (11.4 per cent) produced weak inhibition, and 22 (4.4 per cent) produced inhibition with only the Rh_0'' cell.

This method, like the tube-incubation test for agglutinins, is a valuable index of Rh sensitization. It is now evident that the presence of the blocking antibody does not mean that the individual has undergone desensitization; on the contrary, it denotes a high degree of immunization. The recipient is then likely to have extremely severe reactions from infusion of incompatible blood, and the infants of women whose sera contain these antibodies are as severely damaged, if not more profoundly affected, than those of women whose sera produce strong agglutination by the usual tube test. Moreover, since the presence of this substance denotes a high degree of antibody production and certain disaster to the Rh-positive fetus, repeated quantitative estimations of amounts present in the blood stream are probably more of academic than of clinical importance. This is in contrast to tests for ordinary agglutinins, the quantitative serial estimations of which have proved of considerable value in following maternity cases.

TABLE I. ANALYSIS OF 500 RH ANTISERA

	NUMBER	PERCENTAGE
Sera producing only strong agglutination (in test tube with 2% suspension of red blood cells in saline)	64	12.8
Sera producing only weak agglutination (in test tube with 2% suspension of red blood cells in saline)	201	40.2
Sera producing only strong inhibition (in test tube with 2% suspension of red blood cells in saline)	120	24.0
Sera producing strong agglutination and strong inhibition*	22	4.4
Sera producing weak agglutination with weak inhibition	57	11.4
Sera reacting only on the open slide	36	7.2

*These are sera of the so-called "70 per cent" or anti-Rh′ variety. They contain Rh′ agglutinins demonstrable in the test tube and an antibody which inhibits Rh_0 agglutination. The type of reaction in the test tube, therefore, depends upon the specificity of the red cell against which it is tested.

THE COMBINED TUBE-INCUBATION TESTS FOR AGGLUTINATING AND BLOCKING ANTIBODIES

When the two tests described above are performed in parallel, sensitization can be detected, by careful workers, in most of the cases. The analysis to which we have already referred (Table I) shows that the combined methods indicated sensitization in 464 instances (92.8 per cent). The tests have the disadvantage of being somewhat laborious. They require a good deal of equipment, material, and particularly experience. However, the usefulness of the information they provide offsets the disadvantages.

THE CAPILLARY TUBE TEST

Chown[8] has described an economical and ingenious method for Rh typing which is applicable to the detection of Rh agglutinins. Capillary tubes of 0.4 mm. bore and approximately 8 cm. in length are sterilized in test tubes. One end of the capillary is dipped into the serum to be tested, and a column approximately 2 cm. in height is permitted to run in. The capillary is next dipped into fresh, Group 0, oxalated or citrated blood, making sure there is no air bubble between serum and blood. The tube is then inverted to allow the mixture to run to the opposite end of the tube. The blood-containing end of the capillary is inserted into a plasticine rack at an angle of 45 degrees to the horizontal. After incubation at 37.5° C., the test is read against a white background. With a positive test, a beaded layer is formed along the lower side of the capillary. A negative test produces a thin, smooth line. This method seems to depend on factors similar to those underlying the open slide test (to be described). It is reliable when properly employed, but it may be difficult for the average laboratory worker to read. It also possesses no differential value. Its greatest advantage lies in the fact that it requires a minimum of time, equipment, and material, especially serum. Also, like our slide test, it should detect evidence of Rh sensitization by agglutination, even in the presence of "blocking" antibodies.

THE OPEN SLIDE TEST*

The open slide test was recently described by Diamond and Abelson.[1] Fresh, whole Group 0, Rh-positive blood of normal hematocrit is mixed with about an equal amount of serum (approximately 0.2 c.c.) on the open slide, which is moderately warmed over a 25-watt bulb. The slide is gently tilted from time to time. After a short interval (varying from a few seconds to three minutes), if the test is positive, readily discernible agglutinates appear. A parallel control of Rh-negative blood is highly desirable, particularly in doubtful cases.

Errors in interpretation are chiefly due to nonspecific agglutination or rouleaux formation, which yields a slightly granular appearance to the preparation, but experience helps differentiate this readily from true agglutination, which is much more marked. A drop of saline added to the questionable result with restirring and agitation shows a clear difference between true agglutination and rouleaux formation due to drying.

In the original description[1] it was suggested that once-washed red cells resuspended in saline were satisfactory for performing this test. Further extensive experience has shown that thoroughly washed red cells (two or more times) fail to react unless they are resuspended in plasma or albumin. Only the cells incompletely washed free of their original medium will permit agglutination on the slide. For this reason it is necessary to use oxalated or citrated blood unwashed, or, if washed, resuspended in plasma, serum, or, as recently found most satisfactory, albumin.

At first it appeared that this test was successful because of the fact that sufficient antigen in the form of Rh-positive red cells was provided to absorb "block-

*Dr. A. S. Weiner, to whom this test was demonstrated in April, has confirmed in his own laboratory that even the most highly "blocked" sera produce agglutination by this technique. In his discussion of this test at the conference on Blood Grouping, held at the New York Academy of Sciences, May 18, 1945, he used the term "conglutination," rather than agglutination, for this phenomenon to distinguish it from clumping which occurs with a saline suspension of red cells. Since the "conglutination" reaction between antibody (in serum) and antigen (in the red cells) produces clumping in no way grossly different from the agglutination that occurs in the test tube in saline and since the clumping of Rh-positive cells by the anti-Rh antibody (either of the "blocking" type or not) taking place in vivo has always been designated as *agglutination*, the question might well be raised as to what term should be used in the future. Certainly these observations may make it necessary to change our concept of the agglutination reactions which occur within the body.

ing antibodies'' present in the serum, thus permitting the action of ''masked'' agglutination. This theoretic explanation no longer seems valid.

A discussion of the principles underlying the slide test is presented in another paper.

The following factors appear to accelerate the slide reaction:

The presence of unwashed red cells, or washed red cells resuspended in serum, plasma, or albumin; the presence of an excess of antigen with gentle agitation and mild warming, both of which make clumping more rapid and more easily detectable; the presence of a glass surface. Landsteiner,[9] in discussing nonspecific factors in the second stage of antigen-antibody reactions, wrote, ''The fact that agglutinated cells may stick to the walls of test tubes, and that sensitized cells tend to adhere to leucocytes or to plateles, may possibly be caused, in keeping with the lattice theory, by antibody adsorbed to the glass surface, and perhaps on cells.'' He referred to Mudd's statement[10] that he ''has observed in many years of experience with the interfacial technique that specifically sensitized bacterial and other cells are sticky, not only for each other, but for glass slide and coverslips as well . . .''

A modification of the principles employed in this open slide test method has been adapted by us to test tube use. This consists of the suspension of red cells washed, or unwashed, in plasma, serum, or, as a best medium, human or bovine albumin.* Under these circumstances a concentration of red cells varying from 2 to 5 per cent in such a blood protein diluent can be used. Mixture of one or two drops of this red cell suspension with an equivalent amount of the ''blocked'' serum will yield, after short incubation and centrifugation, visible agglutination. The unknown serum can even be diluted with plasma, serum, or, preferably, albumin and a titration of its strength carried out by this method. In routine use it has been found that the slide test can be carried out more rapidly and yields equally accurate results, so that it is most suitable for use in a large laboratory.

When the precautions mentioned are heeded, the slide test is reliable, simple, and speedy in result. In the Blood Grouping Laboratory it has failed to confirm Rh sensitization only three times in a series of over 1,200 tests; of the 500 cases referred to previously, only one of proved sensitization was missed by this means. An analysis is presented in Table I and a comparison of the three methods in use in our laboratory for detection of Rh sensitization is found in Table II.

TABLE II. COMPARISON OF THREE IN VITRO TESTS FOR DETECTION OF ANTIBODIES IN 500 CASES OF RH SENSITIZATION

	NUMBER	PERCENTAGE
Cases detectable by the tube-incubation test for agglutinins	344	68.8
Cases detectable by the blocking test (often with weak agglutinins)	199	39.9
Cases detectable by the combined agglutination and blocking tests	464	92.8
Cases detectable by the open slide test	499	99.8

From Table II it will be seen that when used alone, neither of the earlier tube tests is as satisfactory as the open slide test for detection of Rh sensitization. The latter test does not, however, indicate specificity of sera, according to Wiener's classification. By its use, no distinction can be made between early and late sensitization, blocking and agglutinating antibodies. Its chief value, therefore, is as a screen test in following obstetric patients, or as a con-

*Details of the study of Rh agglutination using albumin as a diluent are presented in a paper to be published shortly.

firmatory test when tube demonstrations using saline cell suspensions are equivocal. In resolving hemolytic transfusion reactions, we have found it to be of value in detecting incompatibilities other than those due to the Rh factor; in particular, incompatibility between A_2 and 0 or A_1 bloods. For this reason, and because it is in general more sensitive and more reliable than other methods, its use is particularly indicated in dealing with problems involving transfusions and transfusion reaction. More important, it has led to a new understanding of the nature of the "blocking antibodies" so often found in persons sensitized to the Rh blood factor and has suggested new explanations for some of the hitherto puzzling features of many cases of erythroblastosis fetalis. In addition, the demonstration of agglutination of red cells suspended in plasma, serum, or albumin by an antibody which fails to yield visible agglutination of red cells suspended in saline, the time-honored diluent for agglutination tests, has offered a new technique for study. It suggests the need for re-evaluating previously obscure hemolytic reactions. It is a particularly logical technique since within the body, red cells are suspended in blood protein diluents, not in saline alone, and agglutination in vivo may involve not only the red cells and the antibody, but also the blood fluids.

SUMMARY

The various tests available for detection of Rh sensitization are (1) the biologic test, (2) the modified compatibility or tube-agglutination test, (3) the "blocking" test, (4) the capillary tube test, and (5) the open slide test.

The biologic test possesses very limited clinical usefulness and should not be used to supplant, but rather to supplement, the in vitro methods. Of the latter, the open slide test, when used alone, is by far the simplest and most reliable. It is particularly valuable as a screening test in following obstetric patients and as a confirmatory test for compatibility of blood donor and recipient. For a more refined diagnosis, the tube-agglutination and blocking tests used in conjunction with one another possess a high degree of reliability and yield valuable information concerning the stage of immunization to which the sensitized patient has progressed.

REFERENCES

1. Diamond, L. K., and Abelson, N. M.: The Demonstration of Anti-Rh Agglutinins—An Accurate and Rapid Slide Test, J. Lab. & Clin. Med. **30**: 204, 1945.
2. Wiener, A. S., Silverman, I. J., and Aronson, W.: Hemolytic Transfusion Reactions. II. Prevention, With Special Reference to a New Biological Test, Am. J. Clin. Path. **12**: 241, 1942.
3. Wiener, A. S., Wexler, I. B., and Gamrin, E.: Hemolytic Disease of Fetus and Newborn Infant, With Special Reference to Transfusion Therapy and Use of Biologic Test for Detecting Rh Sensitivity, Am. J. Dis. Child **68**: 317, 1944.
4. Levine, P., Katzin, E. M., and Burnham, L.: Atypical Warm Isoagglutinins, Proc. Soc. Exper. Biol. & Med., **45**: 346, 1940.
5. Levine, P., Katzin, E. M., Vogel, P., and Burnham, L.: The Antigenicity of the Rh Blood Factor in Transfusion and Pregnancy—Its Role in the Etiology of Erythroblastosis Fetalis. In Mudd, S., and Thalhimer, W.: Blood Substitutes and Blood Transfusion, Baltimore, 1942, Charles C Thomas, chap. 31, pp. 309-326.
6. Wiener, A. S.: A New Test (Blocking Test) for Rh Sensitization, Proc. Soc. Exper. Biol. & Med. **56**: 173, 1944.
7. Race, R. R.: An "incomplete" Antibody in Human Serum, Nature **153**: 771, 1944.
8. Chown, B.: A Rapid Simple and Economical Method for Rh Agglutination, Am. J. Clin. Path. **14**: 114, 1944.
9. Landsteiner, K.: The Specificity of Serological Reactions, Cambridge, Mass., 1945, Harvard University Press, chap. 7, p. 253.
10. Mudd, S., and Anderson, T. F.: Demonstration by the Electron Microscope of the Combination of Antibodies With Flagellar and Somatic Antigens, J. Immunol. **42**: 251, 1941.

STUDIES IN HODGKIN'S SYNDROME

III. The Relationship of Tubercle Bacilli to Hodgkin's Syndrome

Herman A. Hoster, M.D., Charles A. Doan, M.D., and
Mariellen Schumacher*
Columbus, Ohio

THE possible etiologic relation of the human tubercle bacillus to Hodgkin's syndrome was first suggested by Sternberg[1] in 1898. Twenty-six years later, the recovery of the avian tubercle bacillus by L'Esperance[2] reopened the question of a possible tuberculous etiology in this disease. The present study is an attempted re-evaluation of the etiologic role of human, bovine, and avian tubercle bacilli in Hodgkin's syndrome and is one phase of a broad, general resurvey of the Hodgkin's problem now in progress in this laboratory.[3-5]

In addition to the reports of Sternberg[1] and L'Esperance,[2] a large number of papers have appeared both in support and in refutation of their conclusions. The voluminous literature has been reviewed by Wallhauser (human and bovine tubercle bacillus),[6] Feldman,[7] Branch,[8] and Steiner (avian tubercle bacillus)[9]; see also our recent discussion of the incidence and geographic distribution of cases of Hodgkin's disease reported in the United States of America.[3]

MATERIAL AND METHODS

Six varieties of media were chosen for the attempted isolation of acid-fast organisms:

1. Corper's medium,[10] containing 5 per cent glycerine, malachite green, and Sorenson's phosphate mixture (pH 7.2) (buffer).[11]
2. Corper's medium without buffer and with crystal violet.
3. Egg yolk,† Sorenson's phosphate mixture pH 7.2 (buffer), 5 per cent glycerine, 1 per cent extract of killed human tubercle bacilli and malachite green.
4. Long and Seibert's synthetic medium[12] with 10 per cent serum and malachite green.
5. Petragnani's medium[13] with malachite green.
6. Herrold's medium[14] with malachite green.

It was found that the growth of the two human strains, H 37 and 100.1‡, was most luxuriant on media 1 and 5.

Avian strains 7992, 3746, 3741, and 3296-76§ grew most luxuriantly on medium 3, a modified Corper's medium containing an extract of tubercle bacilli. With these results in mind, media 1, 3, and 5 were chosen for the projected growth of acid-fast organisms from Hodgkin's tissue. Thirty lymph nodes and three spleens were obtained from thirty-three patients with histologically proved

From the Department of Medicine and the Department of Bacteriology, The Ohio State University.

These investigations were made possible in part by contributions from the Elise S. L'Esperance Research Fund and the Comley Research Fund of Ohio State University.

Received for publication, May 13, 1945.

*Elise S. L'Esperance Research Fellow.

†Modification of Corper's medium containing extract of killed human tubercle bacilli.

‡H 37, *Mycobacterium tuberculosis* human. 100.1, *Mycobacterium tuberculosis* human, obtained from the curator of cultures, Department of Bacteriology, Ohio State University.

§7992, *Mycobacterium tuberculosis avium*, obtained from National Type Culture Institute, Georgetown University, Washington, D. C. 3746, 3741, and 3296-76, *Mycobacterium tuberculosis avium*, obtained from Dr. W. H. Feldman, The Mayo Foundation, Rochester, Minn.

Hodgkin's disease for use in the direct inoculation of media. Direct smears stained to demonstrate the presence of acid-fast material were made in each case before the seeding of media. All inoculated tubes and plates were tested at regular intervals for a period of three months whether bacterial colonies appeared to be present or not.

Sixty-six guinea pigs were inoculated subcutaneously in the region of the groin with a thick emulsion of Hodgkin's tissue. Guinea pig controls were similarly inoculated with lymphatic tissue emulsions obtained from fifteen patients with diseases of diverse etiology. Thirty Leghorn chickens were inoculated intravenously with Hodgkin's tissue emulsion, and ten control Leghorn chickens were inoculated with tissue emulsions from other disease sources. Eight rabbits, four control and four experimental, were inoculated in a similar manner. All animals were skin tested with avian O.T. and human P.P.D. tuberculin before inoculation and at intervals during the experimental period. The minimum interval between successive skin testing with avian O.T. was six weeks.

Another group of guinea pigs (twenty in number) was sensitized with 1 to 3 mg. of killed human tubercle bacilli (dry weight) according to the method of L'Esperance[15] eight to twelve days previous to inoculation with Hodgkin's tissue emulsion. Ten additional pigs received only killed tubercle bacilli and were used as controls.

All experimental animals were carefully autopsied either at death or at an elected period eight months following inoculation. An attempt was made in every animal to inject the largest amount of tissue possible in order to obtain the largest obtainable inoculum of acid-fast organisms if present. Animals of both sexes were used in each experimental group. Fixed tissue sections were made from liver, spleen, lung, lymph nodes, and other tissues as indicated. All lesions of a suspicious nature were cultured for acid-fast organisms.

RESULTS AND DISCUSSION

No acid-fast bacilli were isolated either on direct inoculation of artificial media with human tissue inoculum or from autopsy material in the case of the experimental animals, and no macroscopic granulomatous lesions were observed in any of the experimental animals except under the following circumstances:

Four cases in the patient control series were diagnosed histologically as tuberculosis, and in all four cases human tubercle bacilli were obtained from inoculated media and/or from inoculated guinea pigs.

In one of the guinea pigs inoculated with tissue obtained from a patient with Hodgkin's sarcoma there was found at autopsy, eight months after inoculation, the typical macroscopic appearance of advanced Hodgkin's disease of the human liver in miniature. Microscopically, however, the lesion appeared to be fibrous rather than granulomatous and no reticulum cell hyperplasia was present.

Results of skin tests in guinea pigs, chickens, and rabbits were negative except in those cases mentioned in which acid-fast organisms were isolated.

From the results obtained during the present course of study it is not possible to say that the avian, bovine, or human tubercle bacillus, sought under the conditions specified, has an etiologic role in Hodgkin's disease. It is possible, of course, that there may be acid-fast organisms which are difficult or impossible to cultivate on media thus far described; and it is also considered possible that the presence of tuberculo-toxins may contribute in some manner, in perhaps specially sensitized tissues, to the development of the granulomatous lesions observed in Hodgkin's disease. Although it is an established fact that tubercle bacilli may be cultivated at autopsy, in from 15 to 20 per cent of patients with

Hodgkin's disease, and that the immunologic data of Stewart and Doan[16] suggest the not infrequent presence of a concomitant tuberculous process during life, it cannot be concluded that this evidence in any sense establishes a causal relationship between the two diseases. There are many other possible explanations for the coincident presence of the two processes in one individual during life and at post-mortem.

In most cases the tissues studied in the present series were removed because of the necessity for diagnostic biopsy, and the results, therefore, cannot be compared with similar studies in which the material was obtained from patients with advanced Hodgkin's disease or at post-mortem.

SUMMARY AND CONCLUSIONS

A series of chickens, guinea pigs, and rabbits have been inoculated with fresh macerated lymph node and splenic tissue emulsions obtained surgically from patients with Hodgkin's syndrome and other diseases of widely differing etiologies. In each case carefully chosen media were inoculated with tissue emulsion. In no instance were tubercle bacilli found except in the case of histologically, bacteriologically, and clinically proved tuberculosis.

REFERENCES

1. Sternberg, C.: Ueber eine eigenartige unter dem Bilde der Pseudoleukamie verlaufende Tuberculose des lymphatischen Apparates, Ztschr. f. Heilk. **19:** 21-90, 1898.
2. L'Esperance, Elise S.: Primary Splenic Hodgkin's Disease, Proc. New York Path. Soc. **24:** 106-115, 1924.
3. Hoster, H. A.: Studies in Hodgkin's Syndrome. I. A Distribution Study of Hodgkin's Disease in the United States, Ohio J. Sc. **44:** 245-251, 1944.
4. Hoster, H. A., Doan, C. A., and Schumacher, M. E.: Studies in Hodgkin's Syndrome. II. A Search for Brucella in Hodgkin's Syndrome, Proc. Soc. Exper. Biol. & Med. **57:** 86-88, 1944.
5. Hoster, H. A., and Doan, C. A.: Studies in Hodgkin's Syndrome. IV. The Therapeutic Use of Radioactive Phosphorus, J. LAB. & CLIN. MED. **30:** 678, 1945.
6. Wallhauser, Andrew: Hodgkin's Disease—General Review, Arch. Path. **16:** 522-562 and 672-713, 1933.
7. Feldman, W. H.: Avian Tuberculosis Infections, Baltimore, 1938, The Williams & Wilkins Co.
8. Branch, Arnold: Avian Tubercle Bacillus Infection, With Special Reference to Mammals and to Man; Its Reported Association With Hodgkin's Disease, Arch. Path. **12:** 253-274, 1931.
9. Steiner, P. E.: Hodgkin's Disease; Search for Infective Agent and Attempts at Experimental Reproduction, Arch. Path. **17:** 749-763, 1934.
10. Corper, H. J., and Cohn, M. L.: Nutrient Quality of Eggs for Growing Tubercle Bacilli, Am. Hyg. **18:** 1-25, 1933.
11. From Clark, Wm. Mansfield: The Determination of Hydrogen Ions, ed. 2, Baltimore, 1928, Williams & Wilkins Co.
12. Long, E. R., and Seibert, Florence B.: Chemical Composition of Active Principle of Tuberculin; Non-Protein Medium Suitable for Production of Tuberculin in Large Quantity, Am. Rev. Tuberc. **13:** 393-397, 1926.
13. Petragnani: From Kolmer and Boerner: Approved Laboratory Technic, ed. 4, New York, 1945, D. Appleton Century Co., Inc., p. 359.
14. Herrold, R. D.: Egg Yolk Agar Medium for Growth of Tubercle Bacilli, J. Infect. Dis. **48:** 236-241, 1931.
15. L'Esperance, Elise S.: Pathogenicity of Avian Tubercle Bacillus, J. Immunol. **16:** 27-36, 1929.
16. Stewart, F. W., and Doan, C. A.: Analysis of Lymphadenopathy Question With Special Reference to Hodgkin's Disease and Tuberculosis, Ann. Surg. **93:** 141-152, 1931.

STUDIES IN HODGKIN'S SYNDROME

IV. The Therapeutic Use of Radioactive Phosphorus

Herman A. Hoster, M.D., and Charles A. Doan, M.D.
Columbus, Ohio

With the Technical Assistance of Mariellen Schumacher

THE use of radioactive phosphorus in the treatment of Hodgkin's syndrome has been referred to briefly by Low-Beer and co-workers,[1] Hemplemann and associates,[2] and Fitz-Hugh and Hodes[3] in reports devoted to its use in leucemia and so-called allied diseases. The largest series[1] reported (nineteen cases) is representative of other published information on this subject, and the authors concluded that temporary favorable responses occurred only in patients who had "slight manifestations" and that the maximum period of induced remission was one year. Published data on the use of radioactive phosphorus and x-ray therapy in the same patients either concomitantly or successively are so meager that no deductions are justified.

MATERIAL AND METHODS

Radioactive phosphorus used in this study was prepared in the Ohio State University cyclotron under the supervision of Dr. M. L. Pool. Red phosphorus was bombarded with a ten million volt deuteron beam of approximately 100 micro-amperes. The original material was oxidized with nitric acid and the dibasic salt of phosphorus was formed with the addition of sodium hydroxide. The solution was administered in an isotonic form, and its initial activity was approximately 200 microcuries per cubic centimeter. Radioactivity was measured with the Lauritsen electroscope immediately following bombardment.

Eleven patients with Hodgkin's disease, proved by histologic examination of tissue obtained at biopsy, were chosen for study; four were males, and seven were females. The age of each individual and the duration of symptoms before treatment with radioactive phosphorus are indicated in Table I.

A careful evaluation of the clinical status and hematologic equilibrium was made prior to the beginning of the therapeutic period and immediately preceding each subsequent administration of radioactive phosphorus. All dosage is referred to in intravenous equivalents; the effective oral dose is estimated as approximately 75 per cent of the same intravenous dose, the oral route having been used on occasion. Of the eleven patients selected for study, six had received no previous therapy, and five had had previous x-ray therapy. Each biweekly dose was determined as a function of the tolerance of the individual patient, measured in terms of abrupt thrombocytopenia or by other less acute evidences of bone marrow hypoplasia. P^{32} therapy was discontinued and other measures substituted in every case when an increase in the activity of the disease was indicated by additional lymphadenopathy or clinical evidence of bone involvement demonstrable by x-ray.

Parallel studies made in this clinic in an attempt to evaluate the therapeutic

From the Department of Medicine, Division of Cancer Research, The Ohio State University.

Received for publication, May 13, 1945.

TABLE I

CASE	AGE (YR.)	SEX	DURATION OF SYMPTOMS BEFORE P^{32} ADMINISTRATION	DURATION OF P^{32} THERAPY IN WEEKS	BIWEEKLY DOSE IN MILLICURIE	PREVIOUS X-RAY THERAPY	FAVORABLE RESPONSE TO X-RAY AFTER CESSATION OF PHOSPHORUS THERAPY
1	35	F	10 yr.,	21.8	2.27	+	0
2	31	F	1 yr., 2 mo.	13.8	3.01	+	+
3	25	M	5 yr.	14.8	2.22	+	+
4	22	F	1 yr., 11 mo.	13.8	2.48	+	+
5	67	F	3 yr., 10 mo.	21.0	2.26	+	+
6	35	F	7 mo.	21.0	2.55	0	+
7	26	F	1 yr., 1 mo.	28.0	2.77	0	+
8	18	M	2 yr.	10.0	2.49	0	- (Died)
9	39	M	1 yr.	12.7	2.26	0	-
10	29	F	Uncertain	37.0	1.26	0	+
11	46	M	2 yr.	14.8	2.12	0	+
Average value	33.9	7 F 4 M	2 yr., 10 mo.	18.97	2.31	5 positive 6 negative	8 positive 1 negative

TABLE II*

	DECREASE (CASES)	INCREASE (CASES)	UNCHANGED (CASES)
Sedimentation rate	5	5	1
Hematocrit	8	2	1
Hemoglobin	8	3	0
Red blood cells	7	3	1
Platelets	7	2	2
White blood cells	11	0	0
Lymphocytes	6	0	5
Monocytes	6	1	4

*Represents a comparison of hematologic values obtained at the beginning and at the end of radioactive phosphorus therapy.

effectiveness of radioactive phosphorus in leucemia, multiple myeloma, metastatic carcinoma, lymphosarcoma, and erythremia will be presented elsewhere.

RESULTS AND DISCUSSION

In a majority of the eleven patients (Tables I and II) who were treated with radioactive phosphorus, a fall in the hematocrit, in the hemoglobin level, and in total red blood cells, platelets, and total white blood cells, including lymphocytes and monocytes, was recorded during therapy. These changes are similar to those observed by Hemplemann and co-workers[2] in patients with various hematologic dyscrasias who were treated with radioactive phosphorus. There were only two in the series of eleven patients whose sedimentation rates returned to within physiologic limits during therapy (Tables I and II). The most marked and abrupt decline in hematologic values was that recorded in the case of circulating platelets; a platelet decrease from normal or above to a level of 100,000 or less in ten patients, and 50,000 or less in six patients was observed.

The average biweekly intravenous dose of phosphorus was 2.31 millicuries, the minimum being 1.26 millicuries and the maximum being 3.01 millicuries (Table I). Ten of the eleven patients treated developed additional adenopathy or demonstrable evidence of bone involvement during the course of therapy. The maximum and minimum periods during which treatment occurred, before additional evidence of disease activity developed, were thirty-seven weeks and ten weeks, respectively (Table I). One patient (Case 9, Table I) did not return for treatment after the eleventh week. Another patient (Case 8, Table I) died during therapy. The duration of temporary benefit occurring in a few patients cannot be correlated with the duration of the patient's illness at the time of treatment but is probably a function of the rate of activity of the disease at that

time. Eight of the nine patients who received x-ray radiation after cessation of phosphorus isotope therapy responded favorably to the former, as indicated in Table I.

It should be noted that in our experience platelet depression and other signs of marrow hypoplasia did not disappear in most cases after cessation of therapy. Despite the prolonged and severe platelet depression after cessation of P^{32} therapy, no clinical evidence of hemorrhage occurred.

Three representative cases (Figs. 1, 2, and 3—Cases 2, 6, and 7) have been chosen for illustration since they indicate graphically the general trends in hematologic values observed in the group of eleven patients studied.

CASE 7 (Fig. 1).—D. P., a 26-year-old woman, was first seen Aug. 8, 1943, giving a history of progressive and painless left cervical adenopathy of eight months' duration. There was also a history of weight loss of thirty-five pounds, lumbar backache increased during

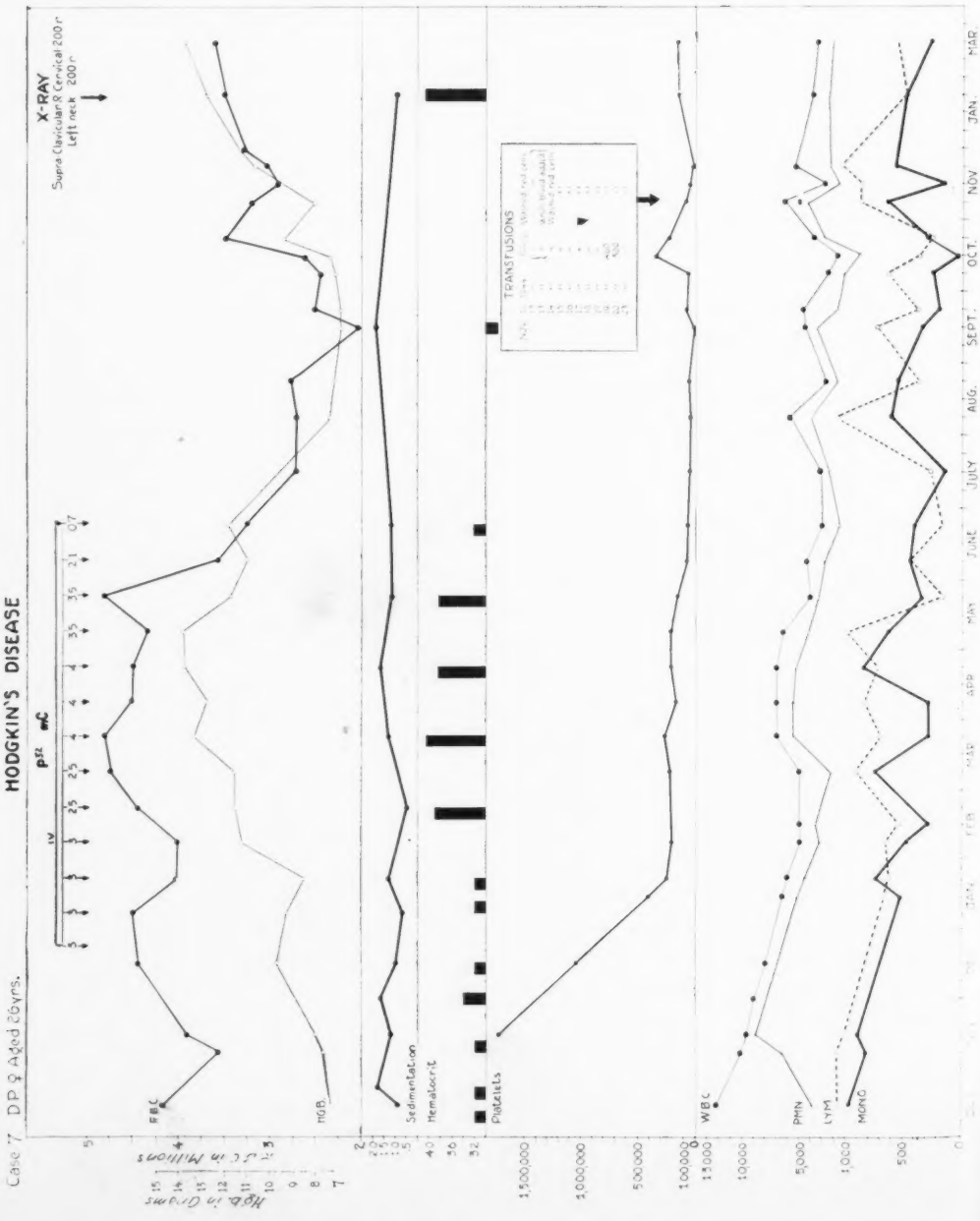


Fig. 1.

menstrual periods, and an increase in nervousness for one year and insomnia, palpitation, weakness, and easy fatigue for six months. The patient had experienced hives (cause unknown) sporadically since childhood and stated that her paternal grandmother had allergic asthma for many years. Physical examination revealed enlarged tonsils and nontender inferior cervical adenopathy 2 by 2 cm. On palpation the enlarged nodes were moderately hard and somewhat adherent to the surrounding tissues. Laboratory studies revealed: White blood cell count 13,350; red blood cell count, 4.34 million; hemoglobin, 11 Gm.; hematocrit, 35; sedimentation rate, 1.3 mm. per minute (Rourke and Ernstene modification of Wintrobe method). The leucocytic differential was as follows: polymorphonuclear neutrophiles, 65; polymorphonuclear eosinophiles, 9; lymphocytes, 19; monocytes, 7; platelets, 1,003,920; and reticulocytes, 1.8 per cent. Urinalysis and serologic tests for syphilis were negative. Biopsy and histologic study of a lymph node obtained from the left inferior cervical region confirmed the clinical diagnosis of Hodgkin's granuloma.

On Nov. 10, 1943, a tonsillectomy was done and the tonsils contained no evidence of Hodgkin's disease either macroscopically or microscopically.

On December 22 radioactive phosphorus was begun as noted in Fig. 1. During the course of P^{32} therapy there was an apparent slight decrease in the left inferior cervical adenopathy two or three weeks after treatment was started. There was, however, no subjective improvement and during the month of April a persistent elevation of temperature of 99° F. was noted. P^{32} was discontinued June 12, 1944, when it was discovered that additional adenopathy in the left superior cervical chain of nodes had developed.

On Oct. 10, 1944, the patient entered the hospital with a complaint of vaginal bleeding of one week's duration. Following a dilation and curettage, bleeding stopped promptly. A diagnosis of functional uterine bleeding was made and multiple washed red blood cell transfusions were given.

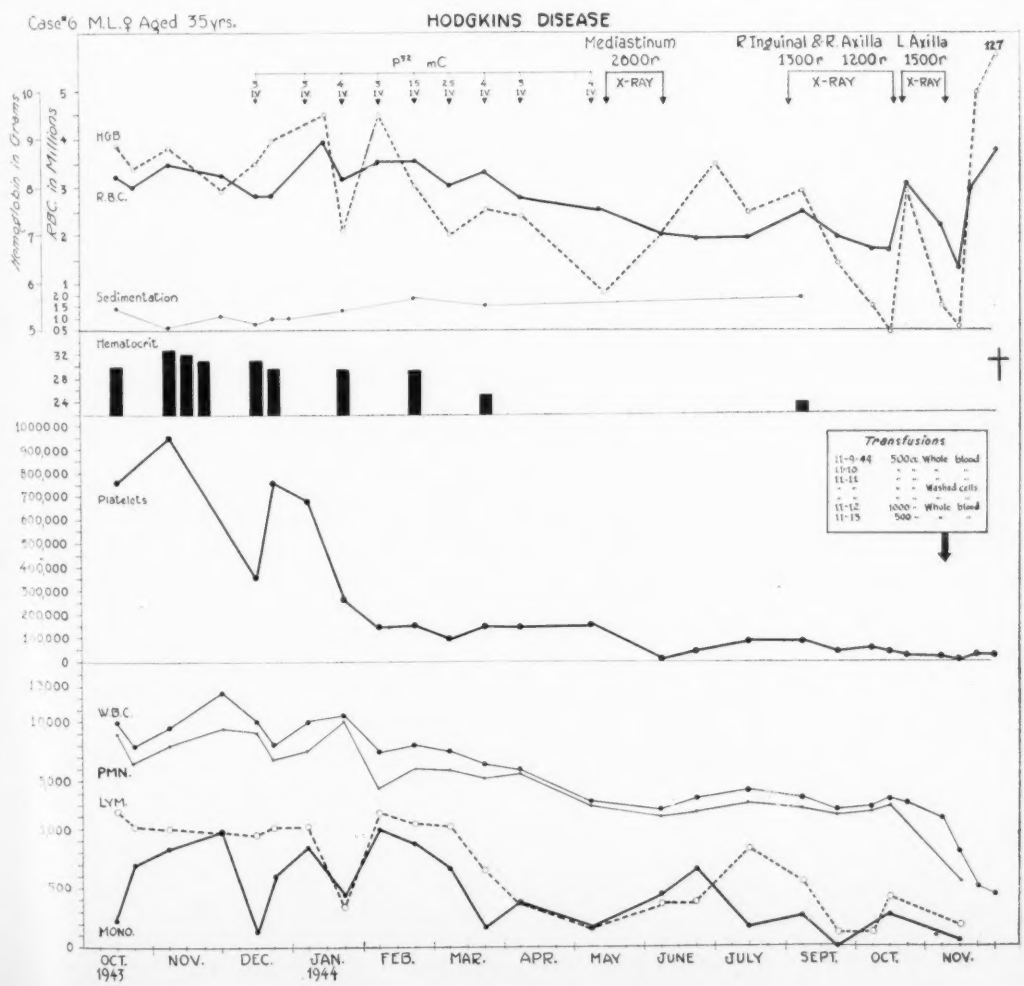


Fig. 1.

Fig. 2.

CASE 6 (Fig. 2).—M. L., a 35-year-old woman, was first seen Aug. 25, 1943, with a chief complaint of painless right cervical adenopathy of four months' duration. There were no abnormal subjective symptoms and no other contributory historical findings. Physical examination revealed no abnormality other than a nontender right inferior cervical adenopathy 1½ by 1½ cm. Laboratory studies revealed the following: white blood cell count, 7,300; red blood cells, 3,600,000; hematocrit, 38; and corrected erythrocyte sedimentation rate, 0.7 mm. per minute. Urinalysis and serologic tests for syphilis were negative. During the next four months the white blood cell count and sedimentation rate rose, the patient became more anemic, and itching of the skin and continuous fever became manifest.

On December 22 radioactive phosphorus therapy was begun as indicated in Fig. 2. On April 25, after four months of treatment and slight subjective improvement in symptoms, a large mass extending from the mediastinum into the upper left chest was observed by x-ray, and an afternoon temperature of 102° F. was recorded. Radioactive phosphorus was discontinued and x-ray therapy begun. The amount and duration of treatment are indicated in Fig. 2.

On October 4 there was a transitory drop in temperature and an increase in the size of the existing nodes. During early November, 1944, the patient developed fluid in the left pleural cavity and despite repeated drainage, rapidly became weaker and died November 15. A profound state of bone marrow exhaustion existed at the time of death. The apparent terminal rise in red blood cells and hemoglobin was produced by multiple transfusions of washed red blood cells.

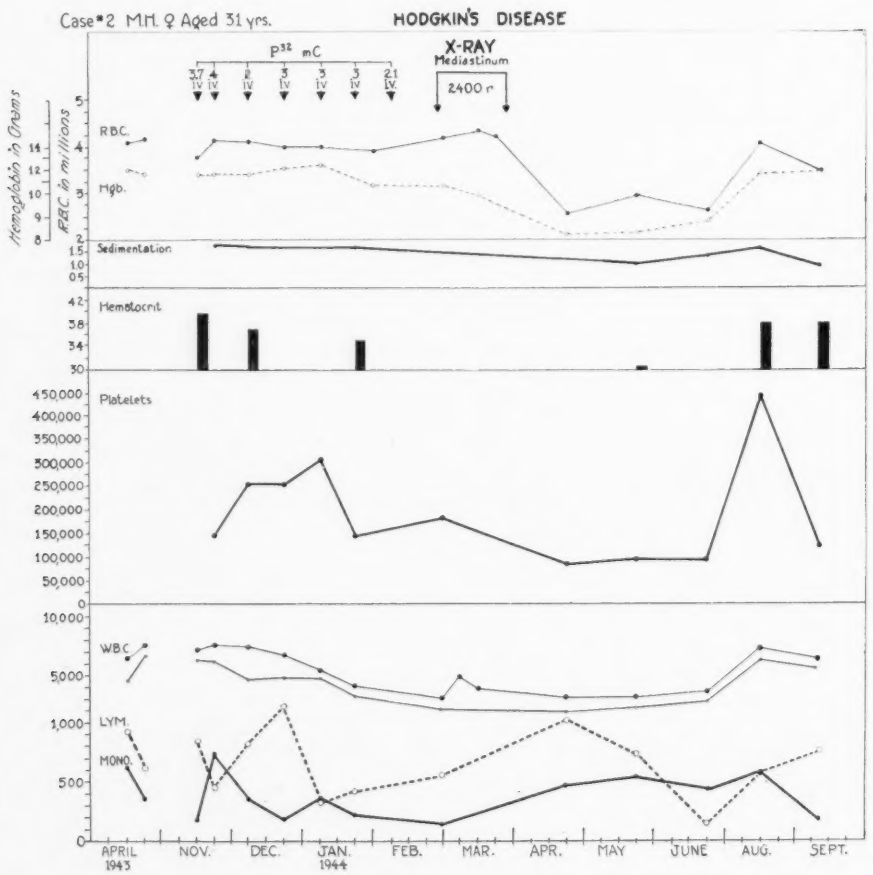


Fig. 3.

CASE 2 (Fig. 3).—M. H., a 31-year-old woman, first seen Jan. 21, 1943, gave a history of having had right cervical adenopathy seven years previously (1936). The adenopathy present at that time was said to have been accentuated by the removal of "infected tubes." Because of the recent change in node size, a biopsy was done and a diagnosis of Hodgkin's disease was made. As a result of this finding, an unknown amount of roentgen therapy was

given over the right cervical region. Oophorectomy, biopsy, and x-ray treatment were carried out elsewhere and complete data regarding them are not available. A recurrence of lymph node enlargement did not occur until six years later (August, 1942), at which time a mass was noted in the lower mid-cervical region. The patient began to complain of weakness, fatigue, nervousness, and numbness in both arms. It was the opinion of the surgeon in charge that the mass was due to thyroid enlargement. At operation (January, 1943) the enlargement was found to consist entirely of Hodgkin's tissue, and following a histologic diagnosis of Hodgkin's granuloma, the patient was referred to this clinic for therapeutic management. A few days after the operation, an eruption with many of the characteristics of erythema nodosum appeared on the lower legs and ankles and disappeared again after a few weeks. Hematologic studies were recorded as follows: hematocrit, 41; sedimentation rate, 1.1 mm. per minute (corrected); white count, 10,500; red cells, 4,110,000; hemoglobin, 11.7 Gm.; supravital differential: polymorphonuclear neutrophiles, 63; polymorphonuclear basophiles, 2; polymorphonuclear eosinophiles, 5; lymphocytes, 19; monocytes, 11.

On Nov. 18, 1943, radioactive phosphorus administration was begun as indicated in Fig. 3. At this time there were palpable a few small left supra-clavicular nodes and a mass 1 by 1 cm. to the right of the sternum and attached to the third rib. One month after phosphorus isotope therapy was started, the patient complained of itching of the skin; after two months of P^{32} administration the patient complained of a burning sensation within the mass to the right of the sternum after each dose of phosphorus was given. After three months of treatment the patient developed a severe cold after which a noticeable increase in the external chest mass occurred and right lateral displacement of the trachea was noted. A few days later difficulty in swallowing became a prominent symptom. Radioactive phosphorus was discontinued and roentgen ray therapy was instituted as indicated in Fig. 3. There was definite clinical improvement noted and the external thoracic mass could no longer be felt, following the completion of x-ray therapy.

SUMMARY AND CONCLUSIONS

The use of radioactive phosphorus in the treatment of eleven selected patients with Hodgkin's disease in the dosage and under the conditions described has not proved of therapeutic value. Depression of hemocytopoiesis, with special emphasis on thrombocytopenia, has been noted.

REFERENCES

1. Low-Beer, B. V. A., Lawrence, J. H., and Stone, R. S.: Therapeutic Use of Artificially Produced Radioactive Substances; Radiophosphorus, Radiostrontuim, Radioiodine, With Special Reference to Leukemia and Allied Diseases, Radiology **39**: 573-597, 1942.
2. Hemplemann, L. A., Reinhard, E. H., Moore, C. V., Bierbaum, O. S., and Moore, S.: Hematologic Complications of Therapy With Radioactive Phosphorus, J. LAB. & CLIN. MED. **29**: 1020-1041, 1944.
3. Fitz-Hugh, T., and Hodes, P. J.: Clinical Experience With Radiophosphorus in Treatment of Certain Blood Dyscrasias, Am. J. M. Sc. **204**: 662-665, 1942.

THE RELATIONSHIP BETWEEN DIET AND THE MECHANISMS FOR DEFENSE AGAINST BACTERIAL INFECTIONS IN RATS

L. Joe Berry, Ph.D., Jane Davis, B.S., and Tom D. Spies, M.D.
Birmingham, Ala.

INTRODUCTION

THE importance of diet in resistance to infectious diseases has long been recognized. A number of controlled animal experiments has shown that such a relationship exists. Watson[1] found a greater number of fatalities in mice fed on a diet from which dried skimmed milk was omitted than in animals whose diet included the milk. Watson, Wilson, and Topley[2] then observed a higher susceptibility to natural contact infection in mice on a diet similar to this than in the animals that were well nourished. The importance of nutrition in nonspecific resistance to infection was clearly demonstrated in mice by Church.[3, 4] Dietary protein has more recently been found by Sako[5] to play an important role in the susceptibility of mice to inoculation with a virulent culture of pneumococci as measured by the postinoculation survival time. Carbohydrates and fats had little influence on this susceptibility. Cannon, Chase, and Wissler[6] depleted rabbits of proteins by restricting the intake and by plasmapheresis and obtained lower agglutinins than in normally fed animals. This was especially true in young animals. Thus, Cannon has emphasized[7] the importance of acquired immunity in resistance to microbic agents and has correlated[8] hypoproteinemia with susceptibility to intercurrent infections in human beings. In Alabama, Riddle, Spies, and Hudson[9] studied 150 patients with pellagra who had definite clinical lesions. *Staphylococcus aureus* and *Streptococcus hemolyticus* were isolated from cheilotic and ocular lesions, and these organisms disappeared following riboflavin therapy. In gingivitis and stomatitis large numbers of Vincent's organisms were found in addition to the bacteria mentioned; these also disappeared following nicotinic acid treatment. In acutely deficient patients a low complement titer and a reduced bactericidal activity of the blood were found. As the dietary improved, these defense mechanisms were returned to normal levels.

Numerous papers in this field have dealt with the relationship of single vitamin deficiencies to specific defense mechanisms. In studies on blood, Abbott and Ahmann[10] have reported a decrease in total white blood cells and in polymorphonuclear cells in rats, and Abbott, Ahmann, and Overstreet[11] found a similar decrease in leucocytes in human beings with clinical vitamin A deficiency. Crimm and Short, on the other hand, found only a neutrophilic index lag in vitamin A-deficient rats[12] and dogs.[13] In cases of pulmonary tuberculosis given large doses of vitamin A, five of ten patients had a left shift and the remainder a right shift.[14] In none of their work did they observe a decrease in total number of white blood cells. Verder and Petran[15] in their study of vitamin A-deficient monkeys observed a slight increase in the average number of leucocytes,

University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Ala. From the Department of Internal Medicine, University of Cincinnati.

The expenses of this study were defrayed by a grant from the Nutrition Foundation and by a grant from the Ralston Purina Company.

Received for publication, May 18, 1945.

polymorphonuclear leucocytes, and erythrocytes. In ten human beings maintained on an A-deficient diet for 188 days, Wagner[16] found a marked leucopenia with a preponderance of degenerate myeloid cells. There was also a thrombocytopenia and a decrease in hemoglobin and erythrocytes.

In 1935, Day, Langston, and Shukers[17] found that the rhesus monkey developed a fulminating fatal blood disease characterized by leucopenia and anemia when fed a diet deficient in B_2 (G). Ulceration of the gums was a consistent accompaniment of the hypocythemia, and diarrhea was common. Several papers have since appeared,[18-20] and it is known that yeast and liver fractions either prevent or cure this disease. Studies on rats fed diets containing from 1 to 2 per cent of one of several sulfa drugs show that severe leucopenia and sometimes anemia may result.[21-25] It can be cured with liver fractions,[21, 23, 24] folic acid concentrates,[21] and crystalline folic acid[23] and improved but not cured by biotin and xanthopterin.[25] The sulfa drugs in nutritional studies are used to alter the intestinal flora of the animal, but results due to toxicity may complicate the natural picture. For this reason the work of György, Goldblatt, Miller, and Fulton[26] and Shukers and Day,[27] in which malnutrition alone has been shown to produce leucopenia in rats, is of special importance. György and co-workers used a diet deficient in vitamin B_6 and found that the leucopenia, thrombocytopenia and anemia which developed could not be cured with vitamin B_6 alone but required Peter's eluate. Shukers and Day fed the animals a riboflavin-deficient diet. Both the deficient rats and the inanition controls became leucopenic, but with the latter group the condition was more severe. Nutritional leucopenia and ulcerative stomatitis in dogs fed a blacktongue-producing diet is reported by Miller and Rhoads.[28] In patients suffering from buccal ulcers a granulopenic leucopenia is frequently encountered.[29]

A reduced phagocytic activity has been reported by Gellhorn and Dunn[30] in undernourished dogs after from six to eight days' starvation. They compared[31] the ingestion of starch granules by washed leucocytes suspended in the experimental serum of each of fifty-seven vitamin A-deficient rats with the ingestion of granules by twenty-two control animals. Thirty of the deficient animals had decreased phagocytosis, and sixteen had increased and eleven had the same phagocytic activity. The severity of the deficiency symptoms did not parallel the changes in phagocytic index. Cottingham and Mills[32] found a decrease in in vitro phagocytic activity of rat granulocytes in animals fed diets with single vitamin deficiencies. This was true for thiamine, riboflavin, pyridoxine, pantothenic acid, choline, singly, and for vitamins A and D combined. No inanition controls were used, but Cottingham and Mills interpreted their results as being dependent upon the general nutritional level of the animals rather than to the effects of the vitamins per se.

The humoral defense mechanism of animals on various types of nutritional regimes has received the attention of several investigators. The tissues of rats deficient in vitamin A were found by Prickett, Miller, and McDonald[33] to have excess lysozyme. In agreement with this observation is the work of Sullivan and Manville,[34] in which the colon of rabbits deficient in vitamin A was found to have four times the lysozyme content as that of controls. In the most comprehensive report of studies on human beings, Feller, Roberts, Ralli, and Francis[35] kept subjects on diets adequate in all respects except for either vitamin A or vitamin C for periods of six, fourteen, nineteen, twenty, or twenty-six weeks. They could find, under these conditions, no influence on the capacity of nasal secretions to inactivate influenza virus, the titer of neutralizing antibodies

for influenza virus, the activity of lysozyme in nasal secretions, complement titer in blood serum, or phagocytic activity for pneumococci of leucocytes in heparinized blood. Negative results were also obtained by Natvig[36] in experimental animals deprived of vitamins A, B, C, or D. He tested, without finding any change, the complement titer, bactericidal power of the blood for dysentery bacilli or *Staph. aureus*, the ability to form agglutinins, and the phagocytic activity. Scaglione[37] has reported a reduction in the agglutinin titers against typhoid and paratyphoid in infants and children deficient in vitamin A. Madison, Fish, and Frick[38] found that nicotinic acid mixed in varying amounts with the antigenic doses of horse serum lowers the precipitin titer in rabbits in small amounts and raises it when given in large amounts. A slight retardation in building up agglutinins against typhoid vaccine was found by Tecce and Visconti[39] in rabbits given nicotinic acid in excess. Jusatz,[40, 41] in a series of experiments with rabbits fed a vitamin-free diet, reports a lowered bactericidal power in blood and a 90 per cent reduction in horse serum precipitins. Vitamins A and D and dried brewers' yeast failed to alter the response. Rose and Kolmer[42] found no change in the complement titer of dogs after four months of B avitaminosis and anhydremia. In fifty persons with vitamin B deficiencies, Morey and Spies[43] showed that the agglutinin titers to *Bacterium tularense* were lower and less well maintained than in normal controls.

Single vitamin deficiency studies are important in elucidating the metabolic function of the vitamins, but single deficiency states seldom occur naturally. For this reason, it was considered of interest to test the various defense mechanisms against infectious diseases in rats fed a diet similar to that upon which persons in an area of endemic malnutrition develop mixed deficiencies, a diet which has been shown to be deficient in many nutrients.[44] Accordingly, a diet for rats was devised, based on the foods eaten by a family studied at the Nutrition Clinic of the Hillman Hospital in Birmingham and characteristic of the diet of many families in this area. It was first used experimentally by Bieler, Johnson, Grant, and Spies,[45] who found that rats suffered loss of weight, loss of appetite, and alopecia, and that normal development was obtained only after the diet had been supplemented with high-grade protein, vitamins, and minerals. Various combinations of the three supplements were found to support growth above the level of the basal diet but considerably below the control level. For this study the supplements which were found to give the best intermediate stages of growth between the basal diet and the diet with all essential supplements were selected from among those used by Bieler and associates. This was done to determine whether resistance to disease could be graded from low to high by dietary means. The results reported here show that this can be done only when the animals are reasonably well nourished and that weight alone is insufficient for predicting the susceptibility to disease.

METHOD

One hundred Sprague-Dawley weanling rats were divided into ten groups, with ten rats in each group. Five groups were males and five groups females. The rats were housed five to a cage. The cages were kept in a steam-heated room, and all animals were treated as uniformly as possible except for diet. They were fed as follows:

Groups 1 and 6	Basal
Groups 2 and 7	Basal + casein
Groups 3 and 8	Basal + casein + minerals
Groups 4 and 9	Basal + casein + B vitamins
Groups 5 and 10	Basal + casein + B vitamins + minerals

The animals in Groups 1 to 5, inclusive, were males, and those in Groups 6 to 10, inclusive, were females. The basal diet consisted of 35.6 per cent corn meal, 28.1 per cent unenriched white flour, 17.6 per cent pork fat, and 18.7 per cent cane sugar. These were thoroughly mixed with sufficient water to make the ration more easily eaten. Eighteen per cent vitamin-free casein* was added to all diets except that for the animals in Groups 1 and 6. The mineral supplement was composed of a mixture of the following salts,[46] which constituted 4 per cent of the diet in Groups 3, 5, 8, and 10:

	GM.
Calcium carbonate	600
Potassium acid phosphate	645
Calcium acid phosphate	124
Magnesium sulfate	204
Sodium chloride	335
Ferric citrate	55
Potassium iodide	1.6
Manganese sulfate	10
Zinc chloride	0.5
Copper sulfate	0.6

The synthetic B vitamins were dissolved in distilled water and added to the other ingredients which then offered the following amounts per day per rat:

	MG.
Thiamine	0.2
Riboflavin	0.2
Pyridoxine	0.2
Inositol	0.2
Ca pantothenate	10.0
Nicotinamide	25.0
Choline	200.0

All the animals received an ad lib quantity of food and the daily consumption for those in each cage was determined by weight difference. This, however, was not an accurate measure since some loss from scattering occurred. Each animal was weighed twice weekly and the average weight per group was calculated. The animals were placed on the diets Sept. 15, 1944, and the final tests were completed Feb. 7, 1945, a total of 146 days. Throughout this time, all rats survived except those lost as a direct result of experimental procedures. There were, therefore, no deaths directly attributable to malnutrition.

A measure of the effectiveness of the three defense mechanisms subject to experimental tests was undertaken. This includes a determination of the total number of leucocytes with differential counts, the phagocytic activity of the granulocytic leucocytes, and the ability to produce antibodies, agglutinins. The only important factor omitted in these experiments is the barrier to invasion by infective agents offered by the skin and mucous membranes. The significance of this barrier in health and disease is not to be minimized, but unfortunately at the present time there is no technique known to us that permits a quantitative estimation of its activity alone. Stryker and Janota[47] found no change in the permeability of the intestinal wall to *Salmonella enteritidis* in vitamin A-deficient rats, but their procedure does not exclude the role of phagocytes.

The tip of the tail was cut in order to obtain blood for erythrocyte counts and white cell differential counts. Bureau of Standards pipettes and counting chambers were used. Wright's stains were made for the differentials. Counts were made on five rats in each of the ten groups.

Phagocytic activity was determined in vitro by the technique of Cottingham and Mills.[32] One-half cubic centimeter of heart blood is diluted in the same

*S. M. A. Corporation, Chicago, Ill.

volume of heparinized* saline and then 0.2 c.c. of a standardized suspension of *Micrococcus candidus* in saline is added. The tube is gassed with a mixture of 95 per cent oxygen and 5 per cent carbon dioxide and shaken for four minutes in a water bath at 38° C. A blood smear is made immediately at the end of the period, stained with Wright's stain, and the number of bacteria is counted in sixty neutrophilic granulocytes. The phagocytic activity of the cells in the five rats in each of the ten groups not used in making blood counts was measured. The final value for each group is thus calculated from a total of 300 white blood cells.

In order to eliminate a possible variation in phagocytosis with time, the blood was withdrawn from all the rats on the same day. Errors that might be attributed to a change in the density of the bacterial suspension due to standing at room temperature were overcome by testing the blood of one rat from each of the five groups of males at the same time and repeating until all had been tested. This was continued without delay throughout the morning hours, and the same procedure was followed in the afternoon for the five groups of females. An aliquot of the same bacterial suspension was refrigerated throughout the morning hours and was then brought to room temperature for use in the afternoon. No consistent variation in phagocytic activity of cells from rats in the same group occurred. Once the smears are stained, the actual microscopic counts can be made over a period of days.

The same rats used for blood counts were originally selected for testing agglutinin production. A phenolized culture of *Bact. tularense* with a density of 7.3 billion cells per cubic centimeter was employed as the antigen.† The animals were injected intraperitoneally at three-day intervals until a total of four injections was given. The first two doses were 0.1 c.c. of vaccine and the last two doses were 0.15 c.c., a total of 0.5 c.c. Two weeks after the final injection, and at biweekly intervals for eight weeks, the males and females were alternately tested for agglutinin titer, but the titers remained very low throughout this time. The serum of the animals in Groups 5 and 10 showed slight agglutination in only the second tube (1:40). Therefore, since this particular antigen gave such a low titer, it was considered desirable to repeat the experiment with a different vaccine.

The five rats most uniform in size that remained in each of the ten groups were placed together in one cage. Each rat was inoculated subcutaneously with 0.2 c.c. mixed typhoid-paratyphoid vaccine.‡ The injections were repeated at intervals of five days until a total of three inoculations was given. Ten days after the immunizing doses had been completed, heart blood was withdrawn and the serum agglutinin titer was determined with the same lot of vaccine for the antigen.

RESULTS

The animals were permitted to remain on the diet for two months before any tests were begun. During this period their weights were checked twice weekly and the growth curves for each group are shown in Fig. 1. After eighty days heart punctures were begun in order to obtain blood for the agglutinin titers, and therefore the death of animals in some of the groups made further records inaccurate. After two months on the diets the animals were photographed, and

*The heparin used in this work was supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

†Supplied by Dr. Lee Foshay, Professor of Bacteriology, College of Medicine, University of Cincinnati.

‡Lederle Laboratories, Inc., Pearl River, N. Y.

their appearance at this time is shown in Fig. 2. There was a subsequent increase in alopecia in the rats in all groups, except those in Groups 5 and 10, but otherwise there was no great change in the general physical characteristics of the animals. A more detailed description of rats fed on these diets may be found in the paper by Bieler, Johnson, Grant, and Spies[45] now in preparation.

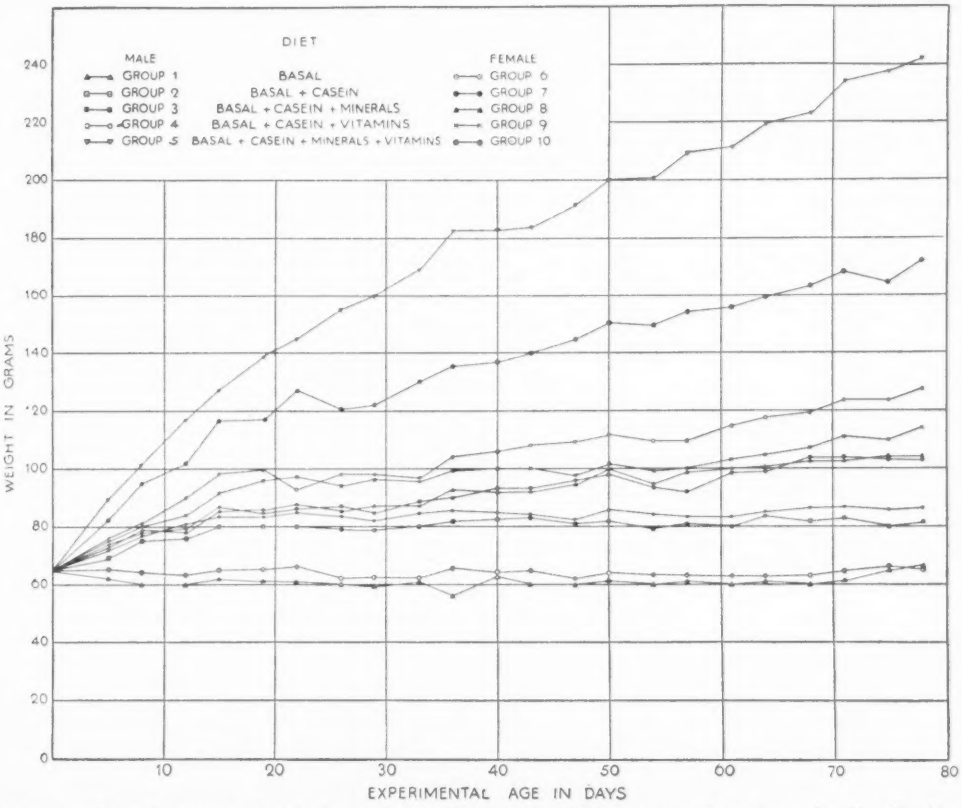


Fig. 1.—Growth curves of rats by groups. Each curve is the average of ten animals. Beginning age is 28 days.

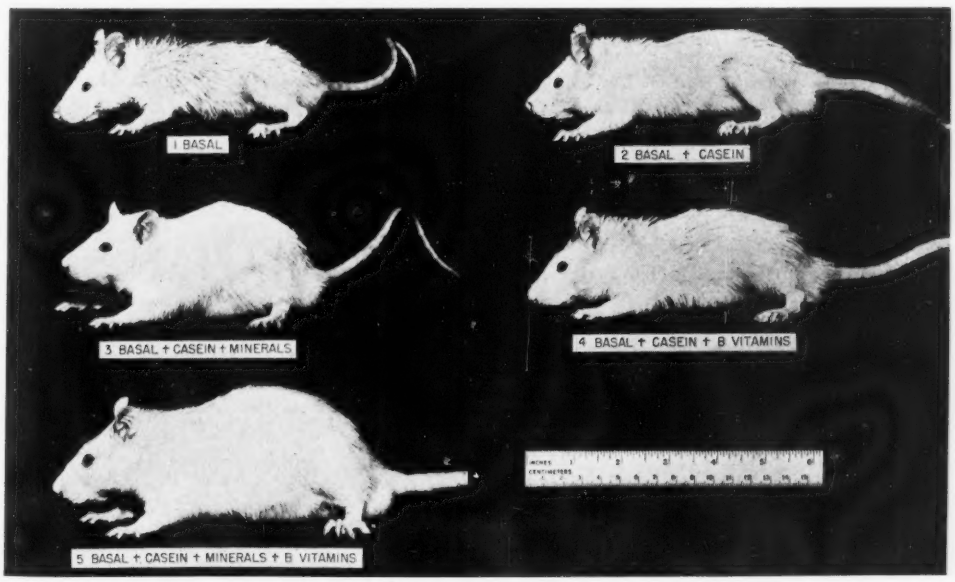


Fig. 2.—Photographs of males from each of the five groups after eighty days on the respective diets. All animals are the same age.

1. *Blood Studies.*—In Table I are summarized the results of the blood studies. Blood counts from the rats in Groups 1 and 6 were obtained after sixty-one days on the diet, and on those in the remaining groups in order (2 and 7, 3 and 8, etc.) during the next ten days until completed. The animals on the basal diet, both males and females, had total white cell counts only about one-third of those in the well-fed, normal groups. The remainder of the groups fell within the normal range for the rat[48]; however, they were all lower than the adequately fed animals. It is interesting to note that the differential leucocyte picture is shifted toward a relative increase in polymorphonuclear neutrophiles with a corresponding relative decrease in lymphocytes and an absolute decrease in all cellular types. This is in agreement with the results reported by Shukers and Day[27] in their blood studies on rats fed a riboflavin-deficient diet with inanition controls. The latter were more severe. No change was observed in the red blood cell totals within this period.

TABLE I. BLOOD STUDIES OF RATS ON DIFFERENT DIETS

GROUP	R. B. C. (IN MILLIONS)		W. B. C.		P. M. N.		LYMPHO.		P. M. E. (PER CENT)	MONO. (PER CENT)
	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE (PER CENT)	RANGE (PER CENT)	AVERAGE (PER CENT)	RANGE (PER CENT)		
1	8.24	7.82-8.59	5,963	5,100-6,650	28.5	20-34	68	62-75	0.5	3.0
2	8.875	8.10-9.90	9,150	6,800-13,000	34	26-48	65	51-73	0	1
3	7.96	7.15-8.95	8,430	6,300-11,350	36	21-44	62	53-74	1	1
4	9.431	9.05-9.775	14,725	12,800-19,450	43	35-49	54	49-62	1	2
5	8.525	8.025-9.325	15,250	12,250-18,500	18	13-22	76	71-81	3	3
6	8.88	7.60-10.45	4,980	2,100-6,550	29	20-36	67.5	60-78	0.5	3
7	9.24	8.65-9.55	10,810	8,600-13,050	34	23-44	63	53-74	1	2
8	8.44	7.75-9.325	7,640	5,000-9,300	25	15-45	72	54-81	1	2
9	8.705	8.05-9.125	11,810	7,700-15,750	27	17-37	70	60-78	1	2
10	8.636	7.60-10.15	14,216	13,000-17,750	21	17-27	75	66-81	2	2

2. *Agglutinin Production.*—In the agglutinin tests against *Bact. tularense* one rat in Group 1 had a titer of 1:320 and one rat in Group 8 had a titer of 1:160. All the remaining animals failed to give complete agglutination in the first tube and showed none at all beyond the third (1:80). The lack of uniformity, the very low titers obtained, and the slowness of response to the antigen made it desirable to test the antibody formation with a different vaccine.

In Table II are shown the results of this study with the typhoid-paratyphoid vaccine. The basal male rats had titers 23.3 per cent as high as the well-fed males, and the females were 45.1 per cent of normal. The three intermediate groups were also greatly inhibited in their ability to form antibodies. There was a spread in the individual titers of rats in each group, but in no case did a deficient rat have a titer as high as the controls. Conversely, the control rats had a more uniform titer, and their lowest titer was equal to, or above, the highest titer of any of the deficient animals.

TABLE II. AGGLUTINATION TITERS OF RATS FED DIFFERENT DIETS

GROUP	AVERAGE TITER	RANGE
1	1:2390	1:320-1:5120
2	1:3718	1:640-1:5120
3	1:2810	1:1280-1:5120
4	1:4120	1:2560-1:5120
5	1:10,240	1:10,240-1:10,240
6	1:7368	1:5120-1:10,240
7	1:6656	1:2560-1:10,240
8	1:7168	1:2560-1:10,240
9	1:9216	1:5120-1:10,240
10	1:16,336	1:10,240-1:20,480

3. *Phagocytic Activity.*—The second column in Table III shows the mean number of bacteria engulfed by neutrophilic polymorphonuclear leucocytes in all rats in each group. These cells in rats on a basal diet were 60.6 per cent for the males and 64.2 per cent for the females as active as those in the control groups. The rats of Groups 2 and 7 and 3 and 8 did not vary significantly from

TABLE III. PHAGOCYTIC ACTIVITY OF ANIMALS ON DIFFERENT DIETS

GROUP	MEAN NUMBER OF BACTERIA PHAGOCYTIZED PER CELL	DIFFERENCE OF MEANS FROM BASAL GROUP	PROBABLE ERROR OF DIFFERENCE × 4
1	8.9400 ± 0.3554		
2	8.2666 ± 0.3719	0.6734	2.0568
3	7.8866 ± 0.3534	1.0534	2.0048
4	11.9400 ± 0.3877	3.0000	2.1037
5	14.7400 ± 0.4235	5.8000	2.2114
6	15.9934 ± 0.2459		
7	14.7560 ± 0.2750	1.2374	1.428
8	14.3320 ± 0.4004	1.6614	1.8796
9	19.0784 ± 0.1566	3.0850	1.1663
10	23.3466 ± 0.3057	7.3532	1.5693

the basal diet animals, while the rats of Groups 4 and 9 were within 74.2 and 81.7 per cent normal, respectively. Column 4 is the calculated probable error of the difference between means, times four. This shows how large the figures in column 3 must be before the difference in means is significant. There is thus no significant increase in phagocytic activity in any of the groups except 4 and 9 and 5 and 10.

The larger number of bacteria phagocytized by the females as compared to the males may or may not be significant. All procedures, as previously mentioned, were carried out on the same day and were designed so as to give a quantitative check between the males in the morning and the females in the afternoon. In spite of the precautions taken, a change in the density of the suspension might very well account for the difference in the results. It was not considered advisable to attempt to check the results by repeating the counts since Mills* has found that bleeding the rats upsets the phagocytic activity so as to prevent a reliable check. Even so, it is interesting to note the close agreement between the percentage variation of the sex groups.

DISCUSSION

These studies support the working hypothesis that resistance to bacterial infections may be depressed by inadequate nutrition. Their importance is enhanced by the fact that the animals were eating the same diet that gives rise to the mixed deficiencies seen in patients at this clinic. The demonstration that antibody production is depressed in rats is in agreement with the observations of Morey and Spies[43] in pellagrins. Also, the fact that granulocytic leucopenia occurred in rats is interesting because for several years we have been studying the incidence of and role of nutrition in leucopenia in people in an area of endemic malnutrition. Furthermore, the lowered phagocytic activity in rats on an inadequate diet is being supported, at least in part, by studies now in progress on the in vitro phagocytosis of the granulocytes of malnourished persons.

It is evident from the fine work of Foster, Jones, Henle, and Dorfman[49, 50] and Rasmussen, Waisman, Elvehjem, and Clark[51] that vitamin deficiencies do not invariably lead to a lowered resistance to infectious diseases. These investi-

*Personal communication.

gations show that with the virus of poliomyelitis, a thiamine-deficient mouse is actually more resistant than the well-nourished mouse. The time for onset of symptoms, their severity, and, to some extent, the mortality rate are all in accord with this fact. The explanation is at present not clear, but an attractive suggestion is that some nutrient essential for the reproduction of the virus is absent. Since the defense mechanisms against bacterial diseases do not seem to function in the same way against many virus diseases, the results reported in this paper need not be considered as contradictory to the above. On the other hand, since the difference in final mortality rate between the various groups of mice studied by Foster and associates is the least significant of all the criteria used in their study, the fatal outcome of the infection was therefore not so strikingly altered by diet. Moreover, the absence of clear-cut results in detecting a difference in agglutinin production with *Bact. tularense* as antigen suggests that the procedure adopted for testing the influence of malnutrition on susceptibility to disease may be important.

The hypothesis that a well-balanced diet is important in maintaining the defenses against infections is supported by this work. Of the rats receiving the various dietary supplements employed in this study, only those (Groups 5 and 10) that received minerals, proteins, and vitamins were normal. Of the rats on the remaining diets, those of Groups 4 and 9 (protein and vitamins) showed a significant elevation of the defense level above that of the rats in the basal group but somewhat lower than the adequately fed rats. Thus, if minerals or vitamins are withheld, the various defenses may be depressed but not in the same proportion as growth. However, it seems unlikely that it is because of a specific functional requirement for such factors alone rather than the extent to which the absence of that factor influences the well-being of the whole organism. Therefore, in mixed deficiencies, the importance of restoring the organism to a balanced nutritional regime becomes apparent if that organism is to be able to defend itself adequately against the onslaught of bacterial infections.

SUMMARY

1. One hundred Sprague-Dawley weanling rats were divided into ten groups with ten rats in each group. The rats in five groups were males and those in the other five groups were females. One group from each sex was fed a basal diet composed of corn meal, unenriched flour, pork fat, and sugar in approximately the same proportion as is eaten by many families seen at the Nutrition Clinic, Hillman Hospital, Birmingham, Ala. The remaining four groups of males and of females were given the basal diet supplemented with casein, casein and minerals, casein and vitamins, and casein, minerals, and vitamins.

2. Growth was recorded and three defense mechanisms against bacterial infections were measured after the rats had been on the diets for a period of from 61 to 146 days.

3. There was a decided decrease in total leucocytes and in all leucocytic elements in the rats on the basal diet after two months. The differential counts showed a relative increase in granulocytes and a relative decrease in lymphocytes in the same animals. Only those rats receiving all three supplements were normal, while the others were intermediate between the two extremes.

4. The males and females on the basal diet had typhoid agglutinin titers only 23 per cent and 45 per cent, respectively, as high as the well-fed groups. Of the other groups, only those animals receiving casein plus vitamin supple-

ments showed an appreciable elevation above these values, and they were only about 50 per cent of normal.

5. The phagocytic activity gave results similar to those for antibody production. Only those animals with the casein plus vitamin supplement had values significantly approaching the activity of the well-fed rats. All other animals were only about 60-65 per cent as active.

6. These observations support the working hypothesis that resistance to certain bacterial infections may be depressed by inadequate nutrition and are in agreement with work previously done with patients. Malnutrition is thus shown to be a factor in susceptibility to infection, but this is not necessarily true in every type of infection.

We wish to acknowledge the assistance of Miss Carol Johnson for her aid in feeding, weighing, and caring for the animals throughout the period of this investigation. We are also grateful to Dr. C. A. Mills for granting us space in his laboratory for housing the animals, for the use of some of his equipment, and for his help and suggestions with the technique involved in making the phagocytic activity measurements.

REFERENCES

1. Watson, Marion: Studies on the Influence of Diet on Resistance to Infection. II. The Effect of Various Diets on the Resistance of Mice to Bacterial Infection, J. Hyg. **37**: 420, 1937.
2. Watson, M., Wilson, J., and Topley, W. W. C.: The Effect of Diet on Epidemics of Mouse Typhoid, J. Hyg. **38**: 424, 1938.
3. Church, Charles F.: Factors Influencing Non-Specific Resistance to Infection, Am. J. Pub. Health **29**: 215, 1939.
4. Church, Charles F.: The Effect of Dietary Minerals on Host Resistance, J. Bact. **34**: 350, 1937.
5. Sako, W. S.: Resistance to Infections as Affected by Variations in the Proportions of Protein, Fat, and Carbohydrate in the Diet, J. Pediat. **20**: 475, 1942.
6. Cannon, P. R., Chase, W. E., and Wissler, R. S.: The Relationship of the Protein Reserves to Antibody Production. I. The Effects of a Low Protein Diet and of Plasmapheresis Upon the Formation of Agglutinins, J. Immunol. **47**: 133, 1943.
7. Cannon, P. R.: Protein Metabolism and Acquired Immunity, J. Am. Dietet. A. **20**: 77, 1944.
8. Cannon, P. R.: Protein Metabolism and Resistance to Infection, Mich. State Med. Soc. J. **43**: 323, 1944.
9. Riddle, J. W., Spies, T. D., and Hudson, N. P.: A Note on the Interrelationship of Deficiency Diseases and Resistance in Infection, Proc. Soc. Exper. Biol. & Med. **45**: 361, 1940.
10. Abbott, O. D., and Ahmann, C. F.: Effect of Avitaminosis A on the Blood Picture, Am. J. Physiol. **122**: 589, 1938.
11. Abbott, O. D., Ahmann, C. F., and Overstreet, M. R.: Effect of Avitaminosis A on the Human Blood Picture, Am. J. Physiol. **126**: 254, 1939.
12. Crimm, P. D., and Short, D. M.: Qualitative Blood Cell Changes in the Rat Due to Vitamin A, Am. J. Physiol. **111**: 397, 1935.
13. Crimm, P. D., and Short, D. M.: Vitamin A Deficiency in the Dog, Am. J. Physiol. **118**: 477, 1939.
14. Crimm, P. D., and Short, D. M.: Qualitative Blood Cell Changes in the Human Due to Vitamin A, J. Indiana M. A. **28**: 175, 1935.
15. Verder, E., and Petran, E.: Vitamin A Deficiency in the Rhesus Monkey. Studies on the Gastro-Intestinal Tract, the Blood and Nervous Symptoms, J. Infect. Dis. **60**: 193, 1937.
16. Wagner, K. H.: Die experimentelle Avitaminose A beim Menschen, Ztschr. f. physiol. Chem. **264**: 153, 1940; correction **265**: 59, 1940.
17. Day, P. L., Langston, W. C., and Shukers, C. F.: Leucopenia and Anemia in the Monkey Resulting From Vitamin Deficiency, J. Nutrition **9**: 637, 1935.
18. Langston, W. C., Darby, W. J., Shukers, C. F., and Day, P. L.: Nutritional Cytopenia (Vitamin M Deficiency) in the Monkey, J. Exper. Med. **68**: 923, 1938.
19. Saslaw, S., Wilson, H. E., Doan, C. A., and Schwab, J. L.: The Vitamin "M" Factor, Science **97**: 514, 1943.
20. Waisman, H. A., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F.: Studies on the Nutritional Requirements of the Rhesus Monkey, J. Nutrition **26**: 205, 1943.
21. Axelrod, A. E., Gross, P., Bosse, M. D., and Swingle, K. F.: Treatment of Leucopenia and Granulopenia in Rats Receiving Sulfaguanidine in Purified Diets, J. Biol. Chem. **148**: 721, 1943.
22. Endicott, K. M., Karnberg, A., and Daft, F. S.: Lesions in Rats Given Sulfathiazole, Sulfadiazine, Sulfanilamide, Sulfamerazine, Sulfapyrazine and Acetylsulfadiazine in Purified Diets, Pub. Health Rep. **59**: 49, 1944.
23. Daft, F. S., and Sebrell, W. H.: The Successful Treatment of Granulocytopenia and Leucopenia in Rats With Crystalline Folic Acid, Pub. Health Rep. **58**: 1542, 1943.

24. Karnberg, A., Daft, F. S., and Sebrell, W. H.: Production and Treatment of Granulocytopenia and Anemia in Rats Fed Sulfonamides in Purified Diets, Science **98**: 20, 1943.
25. Totter, J. R., and Day, P. L.: Xanthopterin in the Treatment of Leucopenia and Weight Loss in Rats Fed Succinylsulfathiazole, J. Biol. Chem. **147**: 257, 1943.
26. György, P., Goldblatt, H., Miller, F. R. and Fulton, R. P.: Panmyelophthisis With Hemorrhagic Manifestations in Rats on a Nutritional Basis, J. Exper. Med. **66**: 579, 1937.
27. Shukes, C. F., and Day, P. L.: The Effects of Inanition and Riboflavin Deficiency Upon the Blood Picture of the Rat, J. Nutrition **25**: 511, 1943.
28. Miller, D. K., and Rhoads, C. P.: The Experimental Production in Dogs of Acute Stomatitis, Associated With Leucopenia and a Maturation Defect of the Myeloid Elements of the Bone Marrow, J. Exper. Med. **61**: 173, 1935.
29. Berry, L. J., Doan, C. A., Welch, J., and Spies, T. D.: Unpublished observations.
30. Gellhorn, E., and Dunn, J. O.: Undernutrition, Starvation and Phagocytosis, J. Nutrition **14**: 145, 1937.
31. Gellhorn, E., and Dunn, J. O.: The Influence of Lack of Vitamin in the Diet on Phagocytosis Promoting Properties of the Blood Serum, J. Nutrition **13**: 317, 1937.
32. Cottingham, E., and Mills, C. A.: Influence of Environmental Temperatures and Vitamin Deficiency Upon Phagocytic Functions, J. Immunol. **47**: 493, 1943.
33. Prickett, P. S., Miller, N. J., and McDonald, F. G.: Lysozyme Studies of Tissues From Animals Deficient in Vitamin A, J. Bact. **33**: 39, 1937.
34. Sullivan, N. P., and Manville, I. A.: Relationship of the Diet to the Self-Regulatory Defense Mechanism; Lysozyme in Vitamin A and in Uronic Acid Deficiencies, Am. J. Pub. Health **27**: 1108, 1937.
35. Feller, A. E., Roberts, L. B., Ralli, E. P., and Francis, T., Jr.: Studies on the Influence of Vitamin A and Vitamin C on Certain Immunological Reactions in Man, J. Clin. Investigation **21**: 121, 1942.
36. Natvig, H.: Die Bedeutung der Vitamine für die Resistenz des Organismus gegen Backerielle Infectionen, Skr. norsk. Vidensk. Akad. I Nat. Naturv. Klasse **2**: 266, 1941; Nutrition Abstr. & Rev. **12**: 1012, 1942.
37. Scaglione, G.: Effect of Vitamin A on the Formation of Agglutinins Against Typhoid and Paratyphoid in the Young Organisms, Boll. d. Inst. sieroterap. milanese **17**: 408, 1938; Nutrition Abstr. & Rev. **9**: 173, 1939.
38. Madison, R. R., Fish, M., and Frick, O.: Effects of Nicotinic Acid on Specific Antibody Production, Proc. Soc. Exper. Biol. & Med. **39**: 438, 1938.
39. Tecce, R., and Visconti, P.: Influence of Nicotinic Acid on Some Indices of Immunity, Boll. Soc. ital. biol. sper. **16**: 183, 1941; Nutrition Abstr. & Rev. **11**: 567, 1941.
40. Jusatz, H. J.: Der Einfluss der Vitamine auf den Immunitätszustand des tierischen Organismus; die fettlöslichen Vitamine A und D, Ztschr. f. Immunitätsforsch. u. exper. Therap. **88**: 472, 1936.
41. Jusatz, H. J.: Der Einfluss der Vitamine auf den Immunitätszustand des tierischen Organismus; wasserlösliche Vitamine, Ztschr. f. Immunitätsforsch. u. exper. Therap. **88**: 483, 1936.
42. Rose, S. B., Kolmer, J. A.: Complement Studies on Dogs During B-Avitaminosis and Anhydremia, J. Infect. Dis. **59**: 126, 1936.
43. Morey, G., and Spies, T. D.: Antibody Response of Persons With Pellagra, Beriberi, and Riboflavin Deficiency, Proc. Soc. Exper. Biol. & Med. **49**: 519, 1942.
44. Spies, T. D., Vilter, R. W., and Douglas, G., Jr.: Nutrition in Convalescence and Rehabilitation, South. M. J. **37**: 560, 1944.
45. Bieler, M., Johnson, C., Grant, J., and Spies, T. D.: Unpublished observations.
46. Hegsted, D., Mills, R. C., Elvehjem, C. A., and Hart, E. B.: Choline in the Nutrition of Chicks, J. Biol. Chem. **138**: 460, 1941.
47. Stryker, Walter A., and Janota, Martha: Vitamin A Deficiency and Intestinal Permeability to Bacteria and Toxin, J. Infect. Dis. **69**: 243, 1941.
48. Griffith, J. Q., Jr., and Farris, E. J.: The Rat in Laboratory Investigation, Philadelphia, 1942, J. B. Lippincott Co., p. 356.
49. Foster, C., Jones, J. H., Henle, W., and Dorfman, F.: The Effect of Vitamin B_1 Deficiency and of Restricted Food Intake on the Response of Mice to the Lansing Strain of Poliomyelitis Virus, J. Exper. Med. **79**: 221, 1944.
50. Foster, C., Jones, J. H., Henle, W., and Dorfman, F.: The Comparative Effects of Vitamin B_1 Deficiency and Restriction of Food Intake on the Response of Mice to the Lansing Strain of Poliomyelitis Virus, as Determined by the Paired Feeding Technique, J. Exper. Med. **80**: 257, 1944.
51. Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F.: Influence of the Level of Thiamine Intake on the Susceptibility of Mice to Poliomyelitis Virus, J. Infect. Dis. **74**: 41, 1944.

TREATMENT OF EXPERIMENTALLY INDUCED TYPE I PNEUMOCOCCUS PNEUMONIA IN ALBINO RATS

A Comparative Study of the Treatment of Pneumonia With Sulfadiazine Alone and in Combination With Sodium Bicarbonate

Elmer H. Loughlin, M.D., Richard H. Bennett, M.D., Julius Wolf, M.D., and Mary E. Flanagan, B.S.
Brooklyn, N. Y.

SINCE the introduction of the sulfonamide compounds, numerous studies on their toxic properties have been made. One of the frequent difficulties encountered with their use has been the occurrence of renal reactions, notably, crystalluria, hematuria, and renal or ureteral obstruction. These reactions were apparently due chiefly to the fact that these drugs, when excreted in an acetylated form, were quite insoluble except in alkaline urine. To produce a pH favorable for their continued solution, sodium bicarbonate was suggested, at first in equivalent amounts, and then when the alkalinity of the urine was found insufficient, doses of four times the amount of sulfonamide drug were employed, that is, 4 Gm. every four hours.[1, 2]

This procedure undoubtedly influences the urinary pH and thereby increases the solubility of the acetylated sulfonamide. However, in pneumonia, significant changes in the metabolism of the body take place, particularly in relation to the electrolytes[3] and plasma volume.[4] In such instances, the use of the large amounts of sodium bicarbonate as recommended could result in further upset of the metabolic processes and act in a "toxic" fashion. Lyons and his associates[5] have shown that increase in plasma volume occurs in normal healthy persons who are given 50 Gm. of sodium bicarbonate over forty-eight hours.

Because of such possibilities, we undertook the solving of two problems: (1) The effect of sodium bicarbonate administered to albino rats suffering from type I pneumonia in amounts relatively equivalent to doses given to human beings, and treated with an accepted potent sulfonamide, sulfadiazine; (2) the blood and urinary changes incident to such medication, particularly the blood carbon dioxide content and combining power and urinary pH. The first is reported in the present paper.

METHODS

Inoculation of the rats was accomplished by spraying small amounts of a pneumococcal culture into both the pharynx and nares, a method described in a previous publication.[6] The culture, consisting of the washings of the peritoneal cavity of a white mouse infected with type I pneumococci (RI strain), was suspended in 16 c.c. of a physiologic saline solution and contained, as a result of the peritoneal inflammation, natural mucin in addition to the other components of an inflammatory exudate. The procedure used to inoculate the

From the Department of Internal Medicine, Long Island College of Medicine.

This work was made possible by a grant from the Winthrop Chemical Company, and the Dr. Frank E. West Fund, established by Miss Elizabeth Frothingham, and the John C. Warren Memorial Fund, established by Dr. Luther F. Warren, in memory of his son.

Received for publication Sept. 12, 1944.

animals was entirely nontraumatizing and, as we have previously stated, simulated closely the manner in which human pneumococcal infections occur.

A total of eighty-two rats, each approximately 300 grams, was thus inoculated. Of these, seventy-seven animals (93.9 per cent) became infected as manifested by blood cultures positive for type I pneumococci. Only those animals developing positive blood cultures were used in this study.

Cultures of the blood were made just prior to the initial treatment, at twenty-four hours after inoculation, at forty-eight hours, and at seventy-two hours, just prior to succeeding treatments. They were then made at daily intervals until death ensued or until no growth was obtained. The blood for culture was plated on Avery's medium for pneumococci, to which was added 5.0 mg. per 100 c.c. of paraminobenzoic acid.

Determinations of free and conjugated sulfadiazine in the blood were made according to the procedure of Bratton and Marshall.[7] A Klett-Summerson photoelectric colorimeter with a No. 54 filter was used.

All animals used, whether dying spontaneously before the fifteenth day or sacrified on the fifteenth day, were autopsied.

Although seventy-seven rats were successfully infected, seventy-four are included in this report, since in three animals the suspensions or solutions of the sulfonamide drug were introduced accidentally into the trachea, producing immediate death. There was, therefore, an unequal distribution of animals in some of the groups.

To insure accurate dosage, the compounds were either suspended or dissolved in distilled water and administered by gavage.

DOSAGE OF SULFADIAZINE AND SODIUM BICARBONATE

A series of nineteen animals, in which no therapy had been given, was used as a control for those receiving treatment. Therapy with sulfadiazine, sodium bicarbonate, and a combination of the two was begun twenty-four hours after inoculation with pneumococci.

Sodium bicarbonate, 500 mg. per animal, was administered to each of twenty animals on three successive days.

Sulfadiazine, 300 mg. of drug per animal, was given to each of sixteen animals on three successive days.

Sodium bicarbonate and sulfadiazine combined, 300 mg. of sulfadiazine and 500 mg. of sodium bicarbonate, was given to each of nineteen rats on three successive days.

RESULTS

Series I (Control Animals).—Sixteen of the nineteen rats in Series I died. The mortality rate was 84.2 per cent. The average period of survival of those dying spontaneously was 6.0 days. Of these, two had pneumonia alone. Eight had, in addition to pneumonia, one complication; meningitis occurred in two, empyema in four, peritonitis in one, and pericarditis in one. Three had two complications: empyema and pericarditis occurred in two, and pericarditis and peritonitis in one. In one, empyema, pericarditis, and meningitis, as well as pneumonia, were found.

Of the three animals surviving fifteen days and sacrificed, two had resolving pneumonia and a third had active pneumonia involving the left lung.

The average duration of bacteremia of the surviving animals was 5.6 days.

Series II (Sodium Bicarbonate).—Seventeen of the twenty animals in Series II died, giving a mortality rate of 85 per cent. The average period of survival of those dying spontaneously was five days. Of these, eight had pneumonia alone. Three had one complication in addition to pneumonia; two rats had pericarditis and one, peritonitis. Five had two complications: peritonitis and meningitis occurred together in two rats, and empyema and pericarditis in three. Meningitis, pericarditis, and empyema complicated the pneumonia in another of the rats that died spontaneously.

Of the three animals sacrificed, one had resolving bilateral pneumonia, and the others presented evidence of active bilateral pneumonia.

The average duration of bacteremia of those sacrificed was 6.3 days.

Series III (Sulfadiazine).—Only one of the sixteen rats in Series III died, giving a mortality rate of 6.2 per cent. This rat died on the eighth day with bilateral pneumonia and empyema; the blood stream had become sterile on the third day.

Of those sacrificed on the fifteenth day, autopsy revealed no pathology in two, resolving pneumonia in seven, fibrinous pleurisy in one, and bronchopneumonic involvement in five.

The average duration of bacteremia of those sacrificed was 4.25 days.

Series IV (Sulfadiazine and Bicarbonate).—There were nineteen rats in Series IV. Death occurred in nine, or 47.4 per cent. Four died on the fourth day, one on the sixth, three on the seventh, and one on the eighth, the average period of survival being 5.67 days. Five of those dying had pneumonia alone. In two, pneumonia was accompanied by one complication (empyema, peritonitis), and the other two rats each had a combination of empyema and pericarditis in addition to the pneumonic involvement. Hematuria occurred in the rat with pneumonia and peritonitis, this animal dying on the seventh day, and in two of the rats with pneumonia alone, both of which died on the fourth day.

Six of the animals sacrificed on the fifteenth day had resolving pneumonia, two had bronchopneumonic involvement, and one had no pathology.

The average duration of bacteremia of those sacrificed was 4.8 days.

SUMMARY AND CONCLUSIONS

1. Type I pneumococcal infection as manifested by positive blood cultures was produced in seventy-four albino rats. In those rats dying spontaneously, pneumonia and, in most instances, such complications as empyema, pericarditis, peritonitis, or meningitis were found at post-mortem examination.

2. Sodium bicarbonate, sulfadiazine, and sulfadiazine in combination with sodium bicarbonate were administered to sixty-six animals, treatment being started twenty-four hours after inoculation.

3. In Type I pneumonia in rats, the administration of sodium bicarbonate alone resulted in a mortality rate essentially the same as in those animals that received no medication at all. However, sodium bicarbonate in combination with sulfadiazine resulted in a mortality rate of over seven and one-half times that obtained with sulfadiazine alone.

4. In the rats receiving sodium bicarbonate alone and in combination with sulfadiazine, the cause of death was pneumonia with a short survival period, whereas in those receiving no sodium bicarbonate, the survival periods were longer and the animals died chiefly of complications.

5. The average duration of bacteremia of the sacrificed animals was longer

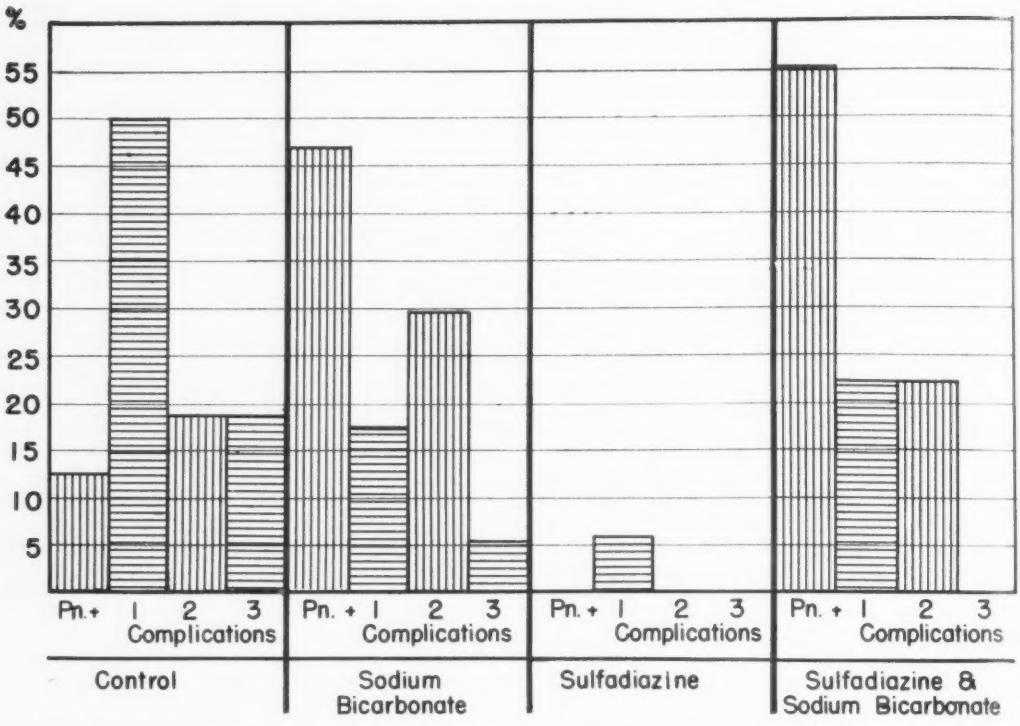


Fig. 1.—Per cent of total mortality due to pneumonia or to its complications. In each group are shown per cent of total deaths, in that group, due to pneumonia (*Pn*), and to pneumonia with one complication (*1*) or with two (*2*) or three (*3*) complications. The complications were empyema, peritonitis, pericarditis, or meningitis.

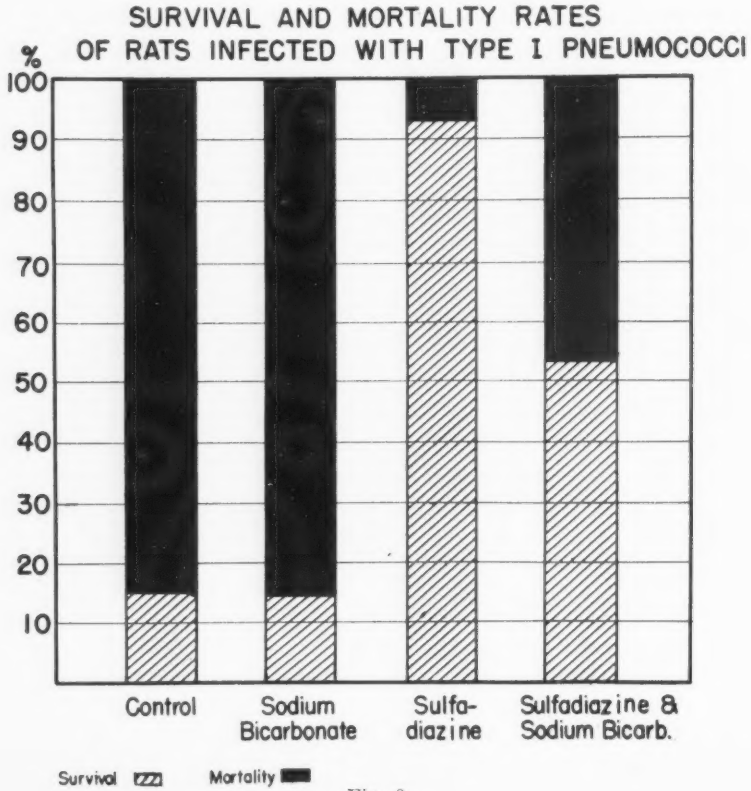


Fig. 2.

in the rats receiving sodium bicarbonate than in those in either of the other groups.

6. Visible toxic reactions as manifested by hematuria were present in 15.7 per cent of the rats receiving a combination of sodium bicarbonate and sulfadiazine as compared with 0 per cent in those in the other groups.

7. Blood sulfadiazine levels were essentially the same in the two groups of animals receiving the drug. The rats receiving the combination of sodium bicarbonate and sulfadiazine had a 15 per cent higher level of free sulfadiazine forty-eight and seventy-two hours after institution of therapy.

The pooled blood of ten rats was used for sulfonamide determinations in each of the two groups. At forty-eight and seventy-two hours, respectively, the free sulfadiazine levels were 10.8 mg. per cent and 12.0 mg. per cent in the rats receiving sulfadiazine and sodium bicarbonate, as compared with 9.5 mg. per cent and 10.8 mg. per cent in those receiving sulfadiazine alone.

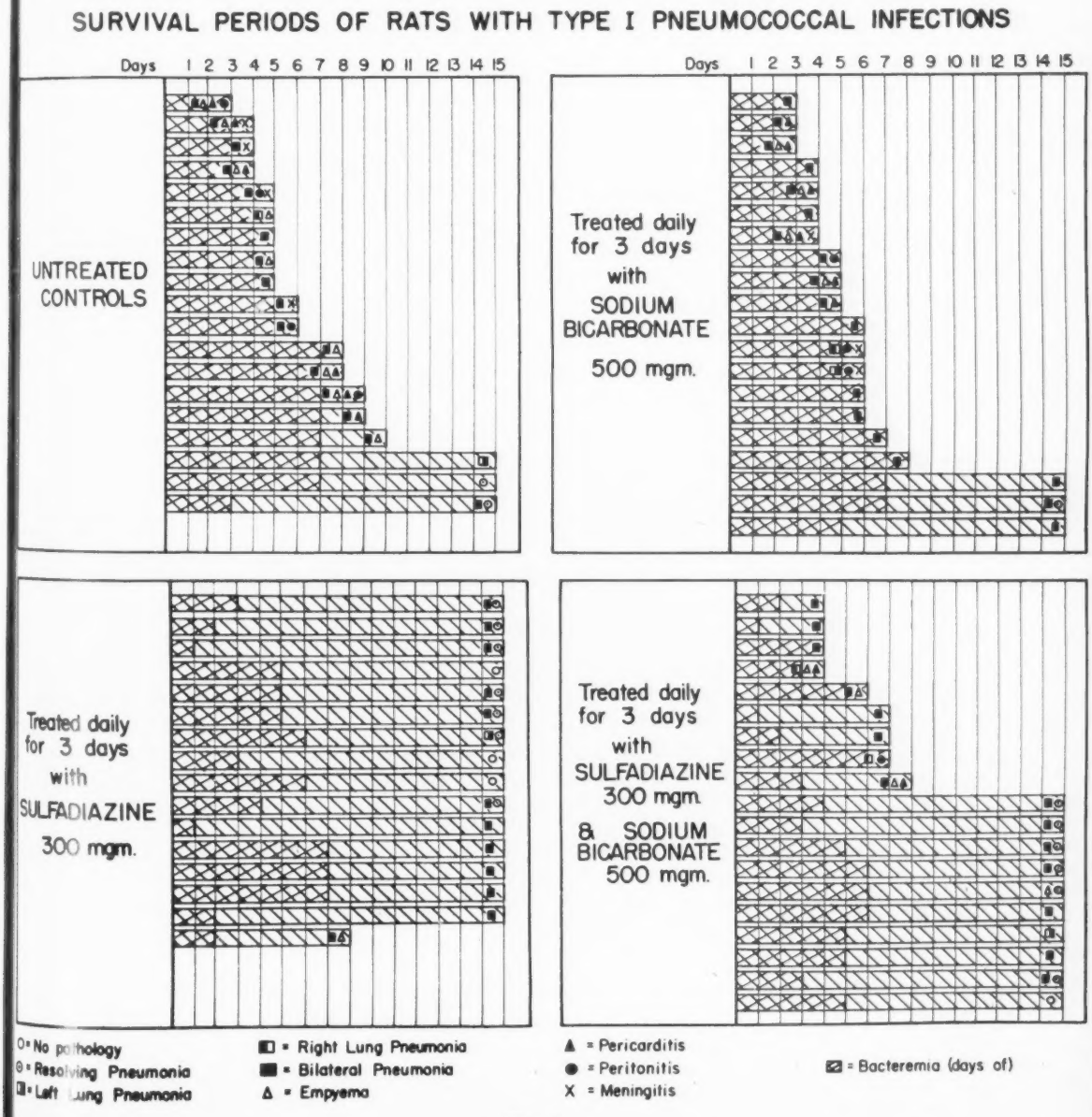

SURVIVAL PERIODS OF RATS WITH TYPE I PNEUMOCOCCAL INFECTIONS
Days 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
UNTREATED CONTROLS
Treated daily for 3 days with SODIUM BICARBONATE 500 mgm.
Treated daily for 3 days with SULFADIAZINE 300 mgm.
Treated daily for 3 days with SULFADIAZINE 300 mgm. & SODIUM BICARBONATE 500 mgm.
O = No pathology
Θ = Resolving Pneumonia
◧ = Left Lung Pneumonia
◨ = Right Lung Pneumonia
■ = Bilateral Pneumonia
Δ = Empyema
▲ = Pericarditis
● = Peritonitis
X = Meningitis
▨ = Bacteremia (days of)

Fig. 3.

CONCLUSIONS

This experiment has shown that in rats sodium bicarbonate exerts a deleterious action in pneumonia. This effect can be seen in the rats receiving only sodium bicarbonate but is more clearly seen in those receiving the combination of sulfadiazine and sodium bicarbonate. We have no experimental evidence, as yet, to offer as an explanation for this phenomenon.

We wish to express our appreciation to Dr. William Dock for his careful criticisms and suggestions in this study.

REFERENCES

1. Fox, C. L., Jr., Jensen, O. J., Jr., and Mudge, G. H.: Prevention of Renal Obstruction During Sulfadiazine Therapy, J. A. M. A. **121**: 1147-1150, 1943.
2. Gilligan, D. R., Garb, S., and Plummer, N.: Prevention of Crystalluria During Sulfadiazine Therapy, Experimental and Clinical Studies; Proc. Soc. Exper. Biol. & Med. **52**: 248-250, 1943.
3. Sunderman, F. W., Austin, J. H., and Camac, J. G.: Studies in Serum Electrolytes. I. Concentration of Electrolytes and Non-Electrolytes in the Serum During Lobar Pneumonia, J. Clin. Investigation **3**: 37-45, 1926.
4. Rutstein, D. D., Thomson, K. J., Tolmach, D. M., Walker, W. H., and Floody, R. J.: Plasma Volume and "Extravascular Thiocyanate Space" in Pneumococcal Pneumonia, J. Clin. Investigation **24**: 11-20, 1945.
5. Lyons, R. H., Jacobson, S. D., and Avery, N. L.: Increase in Plasma Volume Following Administration of Sodium Salts, Am. J. M. Sc. **208**: 148-154, 1944.
6. Loughlin, E. H., Spitz, S. H., and Bennett, R. H.: A Simple Nontraumatizing Method of Inducing Pneumococcic Infection in Albino Rats, J. LAB. & CLIN. MED. **26**: 1181, 1941.
7. Bratton, A. C., and Marshall, E. K., Jr.: A New Coupling Component for Sulfanilamide Determination, J. Biol. Chem. **128**: 537-550, 1939.

THE ACTION OF β-DIMETHYLAMINOETHYL BENZILATE HYDROCHLORIDE, A SYNTHETIC ANTISPASMODIC

HENRY M. LEE, PH.D., CHARLES C. SCOTT, PH.D., M.D., AND K. K. CHEN, PH.D., M.D.
INDIANAPOLIS, IND.

IN THE development of new synthetic antispasmodics, esters of various organic acids and amino alcohols have outstanding possibilities, according to a recent review by Raymond.[1] Blicke and Kaplan[2] prepared a series of fifty-two such esters and found certain ones to possess local anesthetic and mydriatic actions. Furthermore, in this laboratory, tests of some of these esters have revealed antispasmodic activity. One compound in particular, the benzilic acid ester of dimethylaminoethanol,* seems worthy of extensive study because of its high activity. The following report deals with an investigation of its pharmacologic properties.

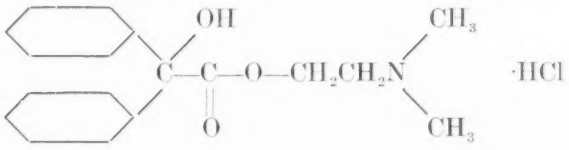
GENERAL PROPERTIES

The hydrochloride of dimethylaminoethyl benzilate is a white crystalline powder, soluble in water, and melts at from 183 to 185° C. When placed on the tongue, it is slightly bitter and produces a sensation of numbness. It is

From the Lilly Research Laboratories, Eli Lilly and Company.
Received for publication, May 12, 1945.

*This substance, first prepared by Blicke, was kindly supplied to us by Mr. H. A. Shonle, of our organic chemical department.

chemically related to trasentin, and its structural formula is shown below. Hereafter, the compound will be referred to by its serial number, HK-141.



ANTISPASMODIC ACTION

Effect on Isolated Organs.—On a pharmacologic basis, antispasmodics may be divided into two groups: (a) those that inhibit parasympathetic motor nerves, as exemplified by atropine, and (b) those which directly depress the smooth muscle fibers, or the papaverine type. Halpern[3, 4] has designated these two classes as neurotropic and musculotropic, respectively. HK-141 appears to possess both types of action.

The musculotropic effect of HK-141 was tested on isolated guinea pig small intestines suspended in a bath of Locke-Ringer's solution. The usual Magnus method of recording was used. Histamine acid phosphate in appropriate amounts was employed to induce spasm of the muscle. After two minutes of histamine stimulation, HK-141 was added to the bath. When the peak action of the latter was attained, the bath was drained and refilled with fresh Locke-Ringer's solution. For comparative purposes, similar tests were run with syntropan and papaverine. Weight for weight, the former was forty to one hundred, and the later, two times, less active than HK-141 in musculotropic action. Concentrations of HK-141 as low as 10^{-4} mg. per cubic centimeter were effective.

By this technique, small intestines of a rabbit were used to demonstrate the neurotropic action of HK-141. In place of histamine, muscle spasm was produced by acetyl-β- methylcholine hydrochloride. On addition of HK-141 to the bath, relief of spasm occurred rapidly. Under these circumstances, the compound was twenty times as effective as syntropan, and about one-half as active as atropine sulfate. The smallest amount of HK-141 which produced this effect was approximately 10^{-5} mg. per cubic centimeter.

The antispasmodic property of HK-141 was not limited to intestinal smooth muscle. Isolated uterine strips of a rabbit, contracting under the influence of epinephrine, were also inhibited by addition of this substance to the bath. In Fig. 1 is illustrated the action of the drug on both isolated uterine and intestinal muscles of rabbits.

Results With Anesthetized Cats.—Using a method similar to that of Bell and Robson,[5] intestinal activity in situ was studied in cats anesthetized with sodium amytal. In place of the photoelectric system, recordings were made directly on a kymograph. Simultaneously, carotid blood pressure was recorded by Hg manometer. Experiments on fourteen animals showed that HK-141 in amounts as small as 0.1 mg. intravenously caused transient relaxation of the gut and depression of peristaltic rhythm. These doses were without effect on blood pressure.

The spasmolytic action was also observed when this compound was given by mouth. To produce a prolonged increase of intestinal activity, cats were injected subcutaneously with physostigmine sulfate about one-half hour before administration of the antispasmodic by stomach tube. Doses of 15 mg. of HK-141 per kilogram, or larger, were effective in relief of the increased intestinal motor activity, whereas effective amounts of syntropan were 75 mg. per kilogram

or greater. Atropine sulfate given by the same route was approximately equal in potency to HK-141. These findings are based on tests made on twenty animals. In Fig. 2 is shown a record of the action of HK-141 when given orally.

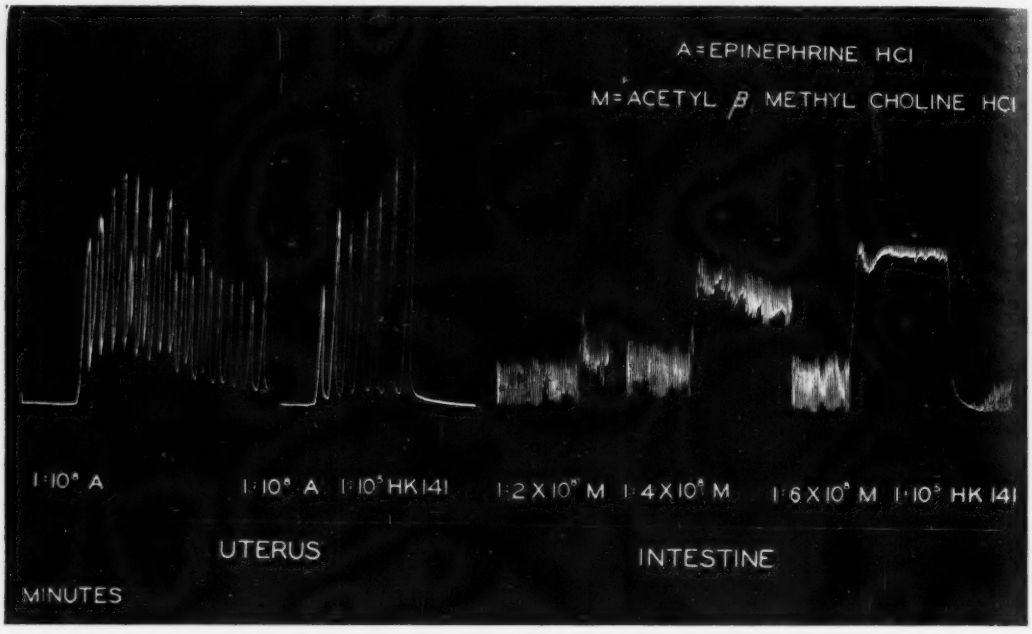


Fig. 1.—The effect of HK-141 on the isolated uterus and small intestine of a rabbit in Locke-Ringer's solution. Stimulation of motor activity of the uterus and intestine was accomplished by epinephrine and acetyl-β-methylcholine hydrochloride, respectively. HK-141 produced a prompt inhibition in each case.

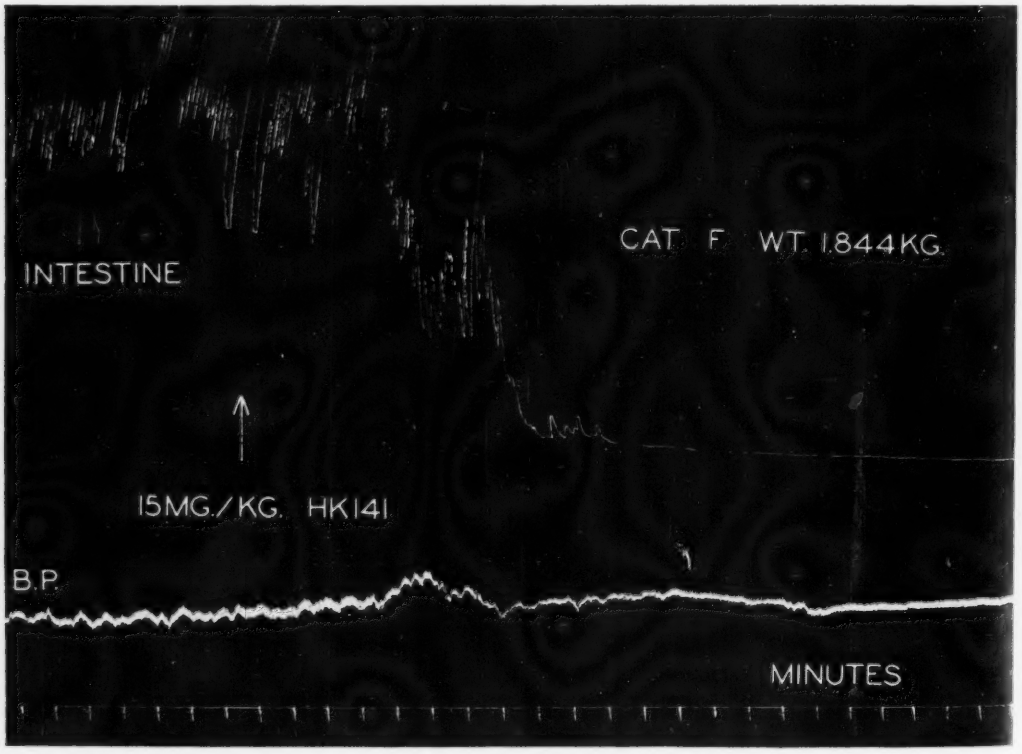


Fig. 2.—The action of HK-141 on intestinal movements in an anesthetized cat. Intestinal motility in situ was recorded by the method of Bell and Robson. At the arrow HK-141 was administered by stomach tube. Note that there was no effect on the blood pressure.

Action in Unanesthetized Dogs.—Three dogs, each with a fistula of the stomach, were trained to lie quietly on a table while gastric motility was recorded by the balloon and water-manometer technique of Carlson.[6] Lehmann and

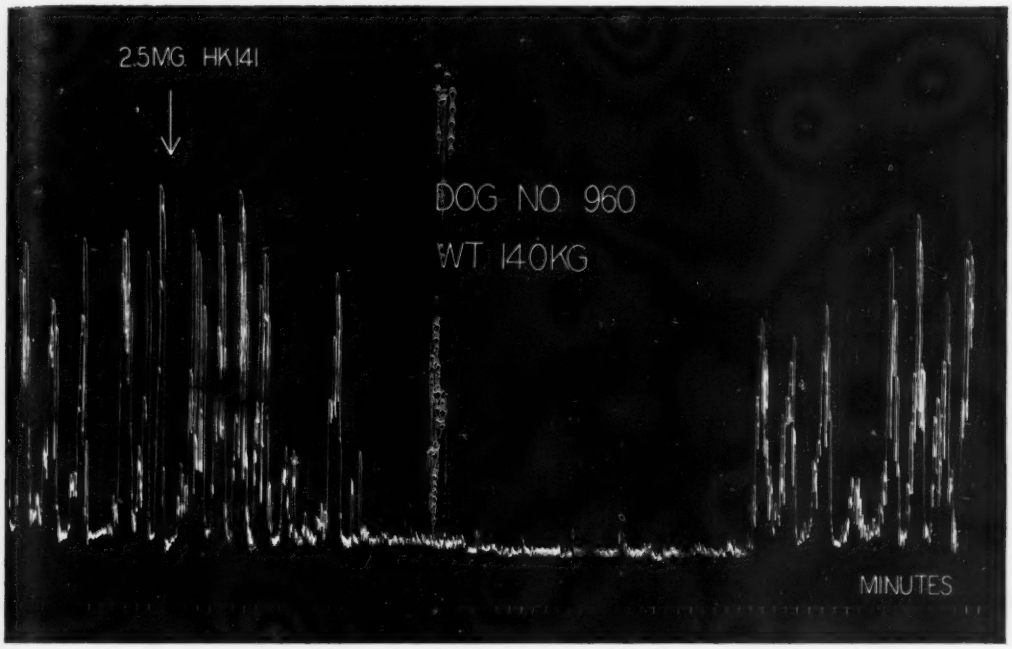


Fig. 3.—The inhibition by HK-141 of hunger contractions in a trained unanesthetized dog. For recording purposes, a balloon connected to a water manometer was introduced into the stomach via a fistula. The arrow indicates a dose of HK-141 administered through the fistula.

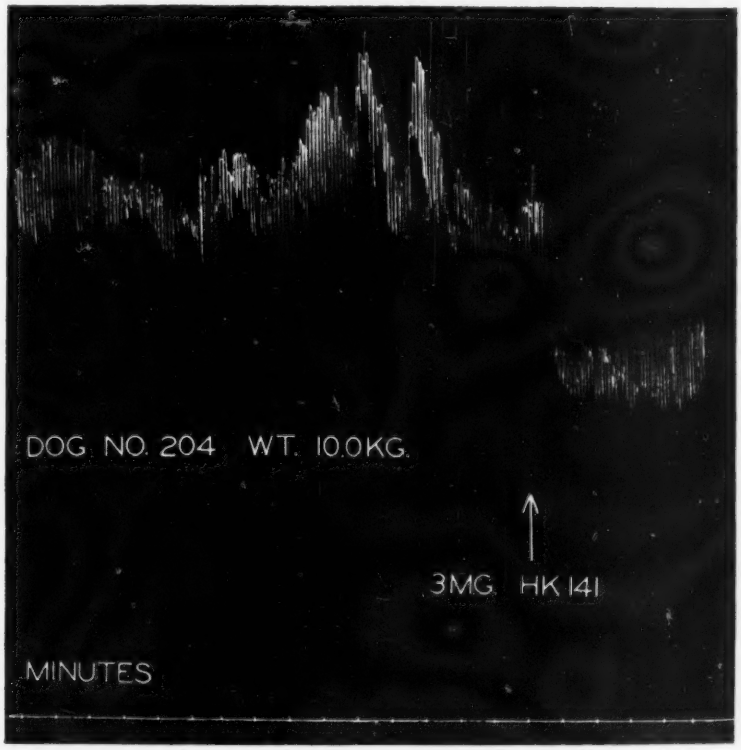


Fig. 4.—Depression of the small intestines by HK-141. Intestinal movements were recorded by a water manometer connected to a small balloon in a Thiry fistula of a dog. HK-141 administered intravenously produced rapid decrease of tone but little or no effect on segmental movements.

Knoefel,[7] in their study of some related compounds, employed a similar procedure, except that they produced gastric hypermotility by insulin injections. In our experience, we found that it was necessary to starve the animals for twenty-four hours only before strong gastric contractions appeared. After starting the record, a hunger period was allowed to proceed until contractions were vigorous. In the middle of the period of hunger contractions, as judged by control experiments, an intravenous injection of from 2 to 5 mg. of HK-141 (total dose) produced prompt inhibition of the hunger contractions, usually of one-half hour's duration. The same effect occurred when the drug was introduced directly into the stomach through the fistula, although under these circumstances, there was a ten- to fifteen-minute latent period. By stomach, the effective dose was 2.5 to 10 mg. (total) and the duration of action about the same as that for the intravenous dose. These results were obtained from a total of eighteen experiments performed on these three dogs. In Fig. 3 is seen a record of the action of HK-141 on hunger of one of them.

The effect of the compound on intestinal motility was tested in five experiments on a dog with a Thiry fistula of the ileum. Means of recording were the same as for hunger; however, a smaller balloon was necessarily used. The intravenous injection of from 0.5 to 10 mg. produced a decrease of tonus of temporary duration. In Fig. 4 is shown a record of this effect. There apparently was little or no action on the segmental movements.

MYDRIATIC AND LOCAL ANESTHETIC PROPERTIES

Solutions of HK-141 in various concentrations were instilled into the conjunctival sac of seven albino rabbits. When the percentage was 0.05 or greater, these solutions produced dilatation of the pupil and inhibition of the light reflex similar to those produced by atropine sulfate. A 1.0 per cent solution similarly applied to the eyes of three rabbits caused anesthesia of the cornea of fifteen to twenty minutes' duration. No evidence of irritation was noted at any time. These results are in general agreement with those of Blicke and Kaplan.[2]

ACTION ON CIRCULATION AND RESPIRATION

In four cats anesthetized with sodium amytal, blood pressure and respiration were recorded in the usual manner. Intravenous doses of HK-141 of 1.0 mg. or less were generally without effect on either blood pressure or respiration. When anesthesia was light, doses of from 10 to 20 mg. caused a moderate stimulation of respiration and a blood pressure fall of from 20 to 80 mm. Hg of a few minutes' duration. Under deep anesthesia, injection of from 5 to 10 mg. resulted in a rise of from 30 to 70 mm. Hg with slight respiratory depression. Doses of from 20 to 40 mg. produced a marked fall of blood pressure and frequently were fatal. Respiratory changes probably were secondary to blood pressure variations, since the former appeared shortly after onset of the latter.

EFFECT ON SALIVARY SECRETION

In a dog anesthetized with seconal sodium, Wharton's duct was cannulated and connected to a drop recorder. In order to obtain a fairly rapid flow of saliva, pilocarpine was injected subcutaneously a few minutes beforehand. Intravenous administration of 0.1 mg. of HK-141 caused a prompt decrease in drop rate from 8 to 2 per minute, with recovery to the original rate in nine minutes. The same dose of atropine sulfate was somewhat more potent, causing depression of drop rate from 8 to 1 per minute and return to original in seventeen minutes.

TOXICITY STUDIES

Acute Toxicity in Mice and Rats.—The median lethal dose ± standard error was determined both by oral and intravenous administration to starved albino mice and rats. For comparison, similar determinations were made with syntropan given intravenously to mice and rats and with atropine sulfate administered by vein to mice. Computations were made according to the method of Bliss.[8] Results are shown in Table I. HK-141 was somewhat more toxic than either syntropan or atropine sulfate. However, the lethal oral dose was quite large for both rats and mice, which may indicate that this substance has a relatively low toxicity. Furthermore, HK-141 is much more active as an antispasmodic than syntropan, so the margin of safety of the former should be greater than that of the latter.

TABLE I. THE MEDIAN LETHAL DOSES (LD_{50}'S) OF HK-141, SYNTROPAN, AND ATROPINE SULFATE IN MICE AND RATS

DRUG	ANIMAL	ROUTE OF ADMINISTRATION	NUMBER ANIMALS USED	LD_{50} ± STANDARD ERROR (MG. PER KG.)
HK-141	Mouse	Intravenous	60	39.9 ± 2.2
		Oral	50	281.0 ± 15.1
	Rat	Intravenous	29	29.9 ± 1.9
		Oral	54	1035.0 ± 35.0
Syntropan	Mouse	Intravenous	35	51.1 ± 2.1
	Rat	Intravenous	26	42.8 ± 2.5
Atropine sulfate	Mouse	Intravenous	36	77.5 ± 3.5

Feeding Experiments in Rats.—Employing the method of Anderson, Henderson, and Chen,[9] young rats weighing about 80 grams were given a diet to which HK-141 had been added. Six groups of five rats each were used, each group receiving a different percentage of the drug. The percentages tested were 0.05, 0.1, 0.2, 0.3, 0.5, and 1.0. Food and drug intakes were recorded daily, while body weights were taken twice a week. The procedure was continued for twenty-eight days. At the end of this period, the rats on the highest percentage were subjected to necropsy. All rats survived the test period, but the 1.0 per cent diet definitely depressed growth. Weight gain was affected very little with concentrations as high as 0.5 per cent. The post-mortem examination of rats whose growth was depressed revealed no pathologic changes. It appeared that HK-141 was relatively well tolerated by rats even in large doses and for prolonged periods.

CONCLUSIONS

The compound β-dimethylaminoethyl benzilate hydrochloride (HK-141) was studied by a number of pharmacologic procedures with the following results:

1. The antispasmodic action of HK-141 is both neurotropic and musculotropic as demonstrated on isolated small intestines. Its ability to antagonize parasympathetic stimulants approaches that of atropine sulfate, while its direct action on smooth muscle is far greater than atropine sulfate and even stronger than papaverine. In both respects, HK-141 is much more potent than syntropan.

2. Isolated uterine strips of a rabbit are also depressed by this substance.

3. HK-141 has an antispasmodic activity equal to atropine sulfate when given by mouth to anesthetized cats.

4. HK-141 in doses of from 2 to 5 mg. intravenously, or 2.5 to 10 mg. orally, inhibits hunger contractions of unanesthetized dogs. Intravenously, from 0.5 to

10 mg. of the compound depress the tone of small intestines of a Thiry fistula dog.

5. The local anesthetic and mydriatic properties of HK-141 have been confirmed in rabbits.

6. Antispasmodic doses of this substance usually have no effect on blood pressure or respiration of cats. Under light barbiturate anesthesia, larger doses cause a fall of blood pressure and respiratory stimulation, while the reverse occurs with deeply anesthestized animals.

7. HK-141 depresses salivary secretion of a dog but is less active than atropine sulfate in this respect.

8. The median lethal doses of HK-141 in rats and mice by intravenous injection are smaller than those of syntropan and atropine.

9. Rats fed the compound in quantities as great as 1.0 per cent of the diet for twenty-eight days show no pathologic changes. This amount depresses growth, but percentages of 0.5 or less have no appreciable effect on weight gain.

The authors are indebted to Mr. R. C. Anderson, Mr. Wallace C. Wood, and Miss Nila Maze for their invaluable assistance in these experiments.

REFERENCES

1. Raymond, A. L.: Recent Developments in Synthetic Antispasmodics, J. Am. Pharm. A. **32**: 249, 1943.
2. Blicke, F. F., and Kaplan, H. M.: Synthetic Mydriatics, J. Am. Chem. Soc. **65**: 1967, 1943.
3. Halpern, B. N.: Sur Quelques Propriétés Pharmacodynamiques d'un Nouvel Ester d'Amino-alcool Synthétique: Le Phenyl-propyl-acétate du Diéthylamino-éthanol, Compt. rend. Soc. de biol. **126**: 678, 1937.
4. Halpern, B. N.: Recherches sur des Esters d'Amino-alcools Doués de Propriétés Spasmolytiques, Arch. internat. de pharmacodyn. et de thérap. **59**: 149, 1938.
5. Bell, G. H., and Robson, J. M.: The Effect of Certain Hormones on the Activity of the Uterine Muscle of the Guinea-pig, J. Physiol. **88**: 312, 1936.
6. Carlson, A. J.: The Control of Hunger in Health and Disease, Chicago, 1916, The University of Chicago Press, p. 42.
7. Lehmann, G., and Knoefel, P. K.: The Effects of Some Spasmolytic Substances on Gastric Function, J. Pharmacol. & Exper. Therap. **74**: 217, 1942.
8. Bliss, C. I.: The Determination of the Dosage-Mortality Curve From Small Numbers, Quart. J. Pharm. & Pharmacol. **11**: 192, 1938.
9. Anderson, R. C., Henderson, F. G., and Chen, K. K.: The Rat as a Suitable Animal for the Study of Prolonged Medication, J. Am. Pharm. A. **32**: 204, 1943.

THE SIGNIFICANCE OF VERY LOW VALUES FOR BLOOD UREA NITROGEN

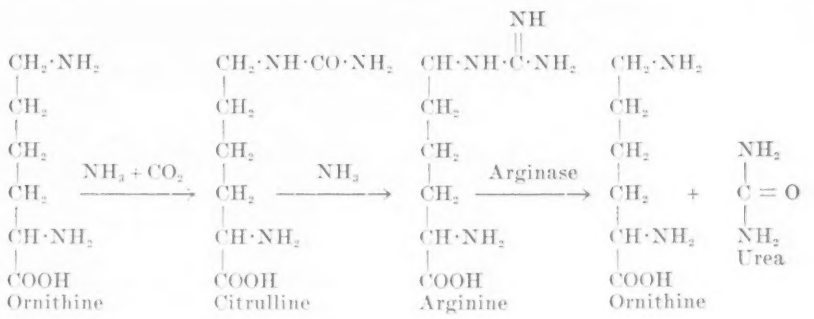
RUSSEL O. BOWMAN, PH.D., FRANCES F. CROWELL, B.A., AND LAURA F. OLSON, B.A.
PROVIDENCE, R. I.

ONE of the end products of protein metabolism in man is urea. This waste product is present in the blood stream and is excreted from the body mainly by the kidneys.

Formation of urea is limited almost entirely to the liver, as shown originally by Bollman, Mann, and Magath,[1] and confirmed by Krebs and Henseleit,[2] with the tissue slice technique. The latter workers proposed the modern con-

From the Biochemistry Laboratory of the Rhode Island Hospital, Providence.
Received for publication, Feb. 19, 1945.

cept of urea formation as due to the combination of carbon dioxide, ammonia from oxidative deamination of amino acids, and ornithine to give citrulline. Citrulline reacts with more ammonia to give arginine, which by the action of liver arginase is broken down to urea and ornithine for repetition of the cycle.



Since Krebs'[3] review in 1936, Evans and Slotin[4] and Rittenberg and Waelsch[5] have shown, by means of isotope studies, that the carbon of urea is derived from carbon dioxide. Schönheimer and Rittenberg[6] have shown that the amidine group of arginine is derived from ammonia, with a similar technique.

Bach and Williamson[7] cast some doubt on the ornithine cycle as the sole mechanism for urea formation, but it is probably the major one.

Once formed, urea does not exchange amino groups with proteins or amino acids. Urinary ammonia is not derived from urea, as formerly taught, but from amino acids[3] by action of the amino acid oxidases of the kidney cortex.

The normal amount of urea in blood of man was determined by MacKay and MacKay[8] by 278 observations on 161 subjects. For males the range was 25.8 to 46.2 mg. per 100 c.c. and the mean 33.0. For females the range was lower, 11.0 to 39.0 mg., and the mean lower at 24.4. Blood samples were usually obtained in the afternoon and their values, for this reason, might be slightly higher than fasting values. They stated that "blood urea of lower than 10 mg. may be considered of questionable normality."

Osterberg and Keith[9] reported twenty-five cases in man of blood urea lower than 10 mg. per 100 c.c. for a four-year period. More females than males were present in the group. Every case was one of severe wasting disease, with either low protein intake or diuresis.

During a period of ten years, while about 80,000 blood urea determinations were done in our laboratory, we have noted many instances of very low blood urea, but we have been unable to explain all of them as Osterberg and Keith did.

METHOD

A modification of the van Slyke and Cullen aeration method was used, as follows:

Two or 3 c.c. of well-mixed oxalated blood, obtained from patients after from eight to twelve hours without food or drink, were pipetted into an eight-by one-inch test tube. One cubic centimeter of a 16 per cent alcoholic extract of permutit-treated Jack bean meal and ½ c.c. of M/2 phosphate buffer at pH 7.0 were added and mixed with the blood and the tube incubated in a water bath at 50° C. for fifteen minutes. About 5 c.c. of distilled water and six drops of caprylic alcohol were added and the tube set up for vacuum aeration into 2 per cent boric acid solution with a methyl-red methylene-blue indicator. The stopper of the blood tube was removed, 5 c.c. of saturated solu-

tion of sodium carbonate solution added, and the stopper replaced. Aeration was slow for five minutes, then at moderate speed for fifty-five minutes. After aeration the boric acid tube was titrated to its original end point (blue) with N/100 sulfuric acid. Final calculation was accomplished by multiplying the titer of N/100 sulfuric acid by 7 for 2 c.c. of blood or by 14/3 for 3 c.c. of blood. This gives a value for blood urea as urea nitrogen in mg. per 100 c.c. It can be converted to milligrams of urea by multiplying by 2.14, although it is our custom to express values as urea nitrogen.

All values to be reported were run in duplicate. Activity of the urease and accuracy of the method were checked with known urea solutions and by recovery of urea added to blood. All such checks proved the method was reliable and accurate.

DATA

From March 1, 1934, until March 1, 1944, among approximately 80,000 determinations of blood urea nitrogen, we have 315 determinations on 276 patients which were below our arbitrarily selected value of 5 mg. per 100 c.c. (equivalent to a blood urea of less than 10.7 mg. per 100 c.c.). In Fig. 1 is shown the incidence of these cases month by month throughout the period. Although there seems to be a higher incidence during 1940 and 1941, more urea nitrogen determinations were being done in our laboratory at that time. There is some correlation, but not a close one, between the total number of determinations of blood urea nitrogen (B.U.N.) and the incidence of very low values.

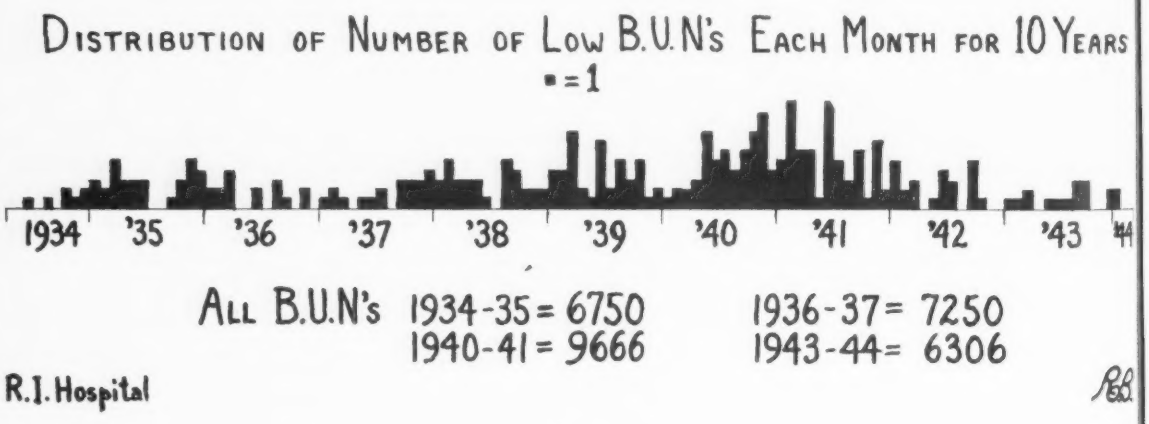


Fig. 1.

Of the 276 cases, 210 were females and 66 were males. The sex distribution of patients in this general hospital is about half male and half female.

In Fig. 2 is shown the age distribution, ranging from 5 months to 79 years. The pattern resembles that of the age distribution of all patients admitted, and no correlation with age is warranted. The average age was 36.3 years.

The distribution of all very low values is shown in Fig. 3. Three instances of B.U.N. of less than 2.5 mg. per 100 c.c. were encountered in three patients. One was in a 28-year-old woman with acute gastroenteritis, who had values of 3.5, 3.0, 4.9, 2.1, 6.3, and 8 mg. per 100 c.c. on two admissions. Another was in a 23-year-old woman with chronic ulcerative colitis, who had values of 2.8, 2.1, 3.5, 5.6, 6, 6.3, 14, 5.6, and 5 during nine admissions. The third was in a 21-year-old woman, in the third month of pregnancy, admitted for ar-

thritis of one hand, who had only two determinations of 2.4 and 7. The first two had poor protein nutrition, while the third was well nourished. The low distribution of cases in the 3.6 to 4.0 mg. section is explained by the fact that most determinations were run on 2 c.c. samples of blood and the titer could be determined only to the nearest tenth of a cubic centimeter, so that values were 3.5 or 4.2, and seldom between these figures.

If we eliminate cases with B.U.N. over 4.6 mg., the limit set by Osterberg and Keith,[9] there are still 185 instances of very low values.

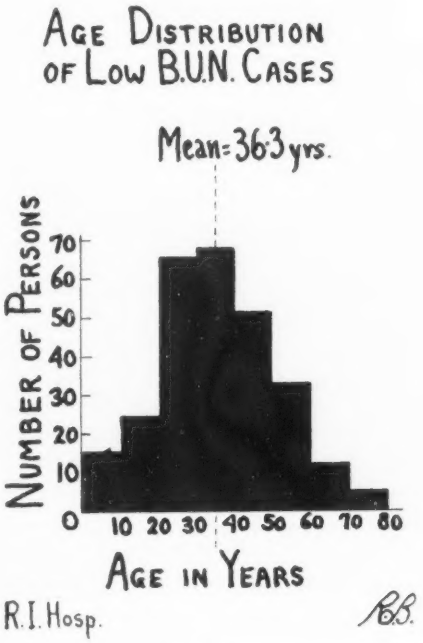


Fig. 2.

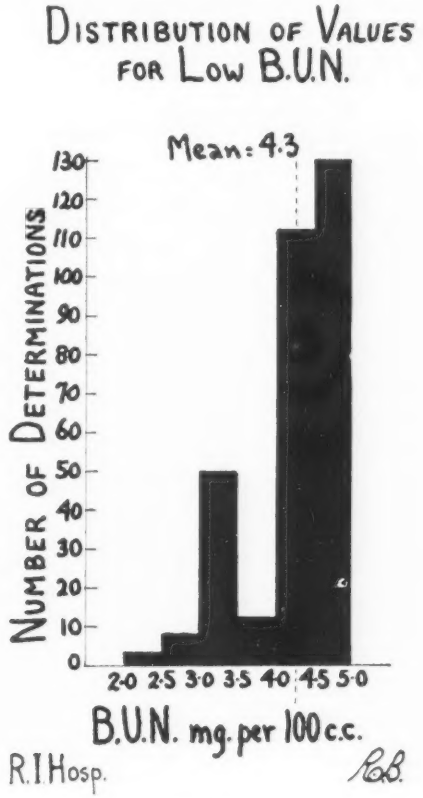


Fig. 3.

DISCUSSION

In an attempt to explain why very low urea nitrogen may be present, the charts of 101 patients, on whom 126 determinations of B.U.N. below 5 mg. per 100 c.c. were obtained, were reviewed. The average age of those in the review group was 37.3 years, ranging from 5 months to 79 years, as for the whole group. There were seventy-two females and twenty-nine males. The mean B.U.N. was 4.1 mg. compared with 4.3 mg. for the whole group. The age distribution and incidence of values were similar to those for the whole group.

Because of Osterberg and Keith's findings, we tried to classify the reviewed cases as chronic wasting disease, chronic protein malnutrition, or active diuresis. Thirty-six were considered chronic wasting disease, with cancer, severe chronic asthma, chronic gastroenteritis, osteomyelitis, or prolonged fevers due to lung abscess, tuberculosis, thrombophlebitis, and endocarditis.

Most of these patients were in chronic protein malnutrition. Nine patients were considered to have protein deficiencies because of dietary habit or lack of intake of protein during postoperative convalescence. Eight patients, other than those mentioned, were in active diuresis for more than twenty-four

hours before the urea nitrogen determination. The remainder, forty-eight patients, were not in diuresis, had had good dietary protein intake, were in good physical condition, and had acute illnesses, or nonwasting chronic ones. As examples, a 52-year-old woman with chronic bronchitis, cor pulmonale, and mild pyelitis had a B.U.N. of 3.5 the day after admission; a 37-year-old woman, admitted for phenobarbital and bromide intoxication, had a B.U.N. of 3.5; a 32-year-old woman had B.U.N.'s of 3.5 and 4.2 on two different admissions for osteomyelitis of the fourth finger of the left hand caused by a human bite; a 59-year-old woman with mild diabetes mellitus and a mild chronic *Escherichia coli* cystitis had one B.U.N. of 3.5, two of 4.9, and twenty-two others ranging from 6 to 25 on three admissions, but showed low specific gravity and urine volume more than 1,500 c.c. previous to only one of the three very low values, a 4.9; a 60-year-old woman with a chronic gastric ulcer history admitted for obstruction had an aluminum hydroxide calculus removed from her stomach when her B.U.N. was 18, and one month later, just before discharge, she had a B.U.N. of 3.5; a 79-year-old woman with acute intestinal obstruction due to a gallstone had a B.U.N. of 27 on admission, one of 7 the day after operation, and one of 4.2 five days after operation, with daily urine volume never exceeding 1,200 c.c.; a 13-year-old boy admitted for acute osteomyelitis of the lower end of the tibia without fever had a B.U.N. of 4.2 twelve days after irrigation and drainage, two others of 6 before and after the 4.2; a 25-year-old man with edema of the legs and dyspnea had a B.U.N. of 4.2 when examined as an ambulatory patient at the cardiac clinic; a 50-year-old man admitted for dermatitis medicamentosa had a B.U.N. of 4.2; a 52-year-old man with second-degree burns of the right arm and shoulder had a B.U.N. of 3.5 on his second day in the hospital.

Dietary or therapeutic factors which might influence the urea nitrogen were looked for, but none were found. The functional capacities of the liver and of the kidney were evaluated in each case. No evidence of liver disease was obtained in 85 of the 101 reviewed cases. In fourteen, there was minimal evidence for liver dysfunction, and in two there was marked liver damage.

Evidence of marked liver damage was based on history of alcoholic intake and ascites, hematemesis, low blood cholesterol, low hippuric acid excretion, and the clinician's opinion. Most of the cases without evidence had only history and physical examination to rule out liver damage. A few had other tests which were negative. None had enlarged liver, jaundice, or history of jaundice, hematemesis, ascites, or poor appetite. One of the cases with marked liver damage also had normal and abnormally high urea nitrogen values. We have seen many other cases of marked liver damage with increased blood urea nitrogen, but only these two cases in the very low range.

The kidneys were presumed to be normal in eighty-three patients, were suspected of poor function in seventeen, and were markedly damaged in one. Osterberg and Keith[9] had one low urea in a patient who died later in uremia. Our patient had carcinoma of the cervix, grade III, bilateral pyelitis, and acute perinephritis. Her urea nitrogen rose from 15 to 36 before nephrostomy and abscess drainage, then fell to 5.6, rose to 17, and was 4.2 a few days before discharge with bilateral nephrostomy. She expired four months later at home.

Ten of the 101 patients died. Four were dead more than one day, but less than seven days, after the low urea nitrogen determination. Six others died more than seven days after the analysis. Thus the finding of a very low urea nitrogen is not a pre-mortem change. Schmidt and Carey[11] have noted an increase in urea nitrogen just prior to death.

We have no explanation for the higher incidence of females than males. The incidence is of the same order as that by Osterberg and Keith,[9] and MacKay and MacKay[8] found lower values in normal women than in normal men.

Although Osterberg and Keith[9] were able to explain their cases, we find about one-half of ours without explanation. We have many examples of chronic wasting diseases with poor protein intake, and with diuresis, yet with normal or even abnormally high urea nitrogen. There is no certainty that these factors will give very low urea nitrogen values. We are more inclined to believe that such instances are rare and are explained by temporary increases in kidney excretion of urea. Since urea is an important diuretic by itself, low blood urea would tend to decrease its diuretic action and lead to higher (normal) values later.

SUMMARY

Three hundred fifteen instances of blood urea nitrogen of less than 5 mg. per 100 c.c., in 276 hospital patients, were found in ten years among 80,000 analyses.

The very low figures for urea nitrogen were not found in any age group or correlated with diagnosis or therapy.

Very low figures were obtained three times as often in females as in males.

The blood urea nitrogen reaches a low of 2.1 mg. per 100 c.c. Values as low as 2.4 mg. per 100 c.c. may be obtained without evidence of diuresis, poor protein nutrition, or dysfunction of the liver.

REFERENCES

1. Bollman, J. L., Mann, F. C., and Magath, T. B.: Studies on the Physiology of the Liver. VIII. Effect of Total Removal of the Liver on the Formation of Urea, Am. J. Physiol. **64**: 371, 1924.
2. Krebs, H. A., and Henseleit, K.: Untersuchungen über die Harnstoffbildung im Tierkörper, Ztschr. f. physiol. chemie **210**: 33, 1932.
3. Krebs, H. A.: Metabolism of Amino Acids and Related Substances, Ann. Rev. Biochem. **5**: 247, 1936.
4. Evans, E. A., Jr., and Slotin, L.: The Role of Carbon Dioxide in the Synthesis of Urea in Rat Liver Slices, J. Biol. Chem. **136**: 805, 1940.
5. Rittenberg, D., and Waelsch, H.: The Source of Carbon for Urea Formation, J. Biol. Chem. **136**: 799, 1940.
6. Schönheimer, R., and Rittenberg, D.: The Study of Intermediary Metabolism of Animals With the Aid of Isotopes, Physiol. Rev. **20**: 218, 1940.
7. Bach, S. J., and Williamson, S.: Formation of Urea in the Mammalian Body Without Participation of Arginase, Nature **150**: 575, 1942.
8. MacKay, E. M., and MacKay, L. L.: The Concentration of Urea in the Blood of Normal Individuals, J. Clin. Investigation **4**: 295, 1927.
9. Osterberg, A. E., and Keith, N. M.: The Clinical Significance of a Very Low Concentration of Urea in the Blood, J. Lab. & Clin. Med. **20**: 141, 1934.
10. Van Slyke, D. D., and Cullen, G. E.: The Determination of Urea by the Urease Method, J. Biol. Chem. **24**: 117, 1916.
11. Schmidt, E. G., and Carey, T. N.: Terminal Hypoglycemia, Arch. Int. Med. **47**: 128, 1931.

LABORATORY METHODS

A PROTECTIVE CABINET FOR INFECTIOUS DISEASE LABORATORIES

C. C. Shepard,* C. W. May,† and N. H. Topping‡
Bethesda, Md.

SEVERAL procedures commonly employed in the investigations of certain of the infectious diseases are known to be dangerous. With the advent of the use of the embryonated egg for the growth of infectious agents it has seemed that laboratory infections have become more frequent. The membranes and other tissues of the egg at times are exceedingly rich in the infectious agent and often the procedures designed to disrupt these tissues tend to disperse finely divided droplets into the air. The Waring blender is an example of one such piece of laboratory equipment extensively used for the grinding of tissues that will on occasion leak and disperse infected material into the air.

Van den Ende§ has indicated the dangers inherent in intranasal inoculations of small animals. He has published a description of a box designed to protect the investigators engaged in such a procedure. The salient feature of this protective device consists of a constant draft produced by a gas burner with incineration of the contaminated air.

Using the basic principle of the box described by Van den Ende, we have designed a protective cabinet to house electrical equipment which may be a source of danger. The important detail of this cabinet is a guillotine type window which, when open more than 1/4 inch, throws an electrical limit switch, cutting off the power to the equipment inside.

Description of the Protective Cabinet.—The cabinet is constructed of wood and is made as nearly airtight as possible (Figs. A and B and Diagram 1). The floor is 30 by 30 inches, the sides are 24 inches in height, and the tapered top is approximately 12 inches in height and leads to the exhaust stack. The front consists of a snugly fitting vertical sliding window, counterbalanced by a sash weight and equipped with an electric limit switch to control power to the electrical outlets located inside the cabinet. There is mounted in the cabinet an ultraviolet light and an electric light for illumination, each of which is connected to an independent snap switch not in the circuit with the limit switch on the window. A baffle, 8 3/4 inches square, of ordinary transite is inserted at the top of the box a short distance beneath the stack to interfere with the transmission of radiant heat from the stack.

The stack is 43 inches in overall height and 7 inches in diameter. It is composed of three sections: (A) a 6-inch length of galvanized sheet metal stovepipe which is fastened to the top of the cabinet; (B) the heater section of 3/32 inch black metal 18 inches long which contains the burner (Diagram 1,

From the Division of Infectious Diseases, National Institute of Health.
Received for publication, May 9, 1945.

*P. A. Surgeon, United States Public Health Service.

†Administrative Assistant, U. S. Public Health Service.

‡Surgeon, United States Public Health Service.

§Van den Ende, M.: An Apparatus for the Safe Inoculation of Animals With Dangerous Pathogens, J. Hyg. 43: 189, 1943.

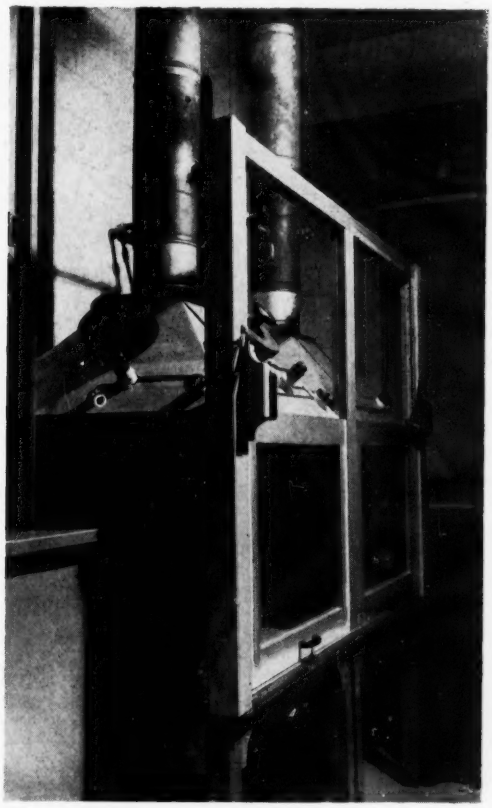


Fig. A.—Exterior view of two of the cabinets.

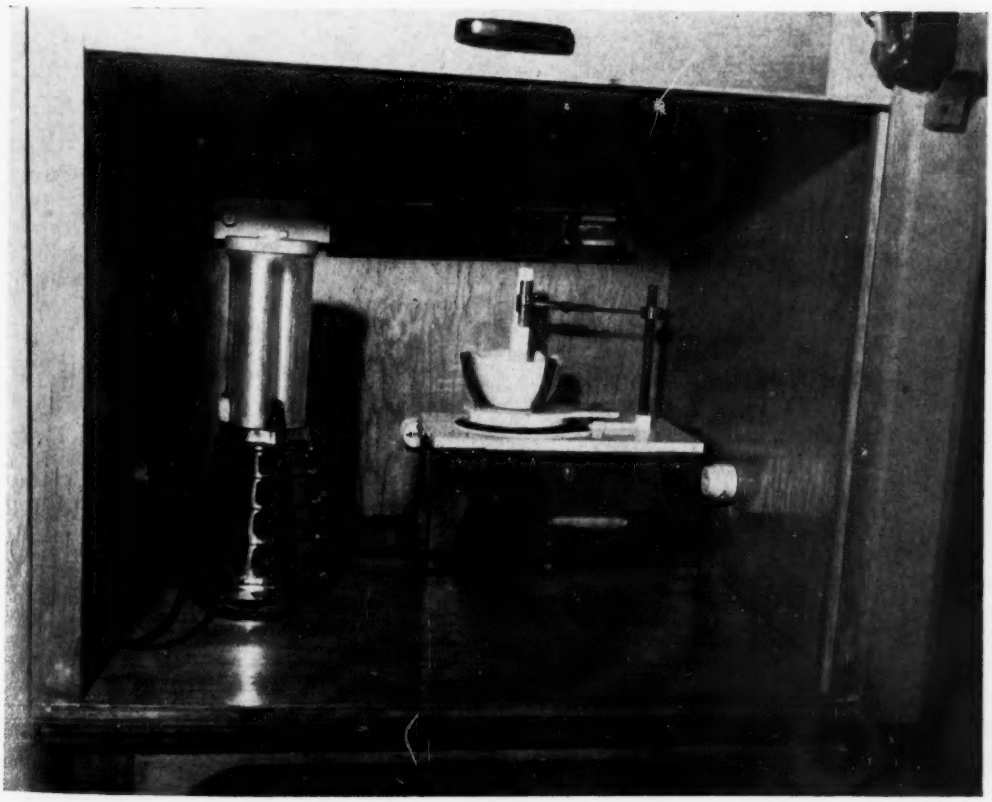


Fig. B.—Interior view of one of the cabinets with equipment.

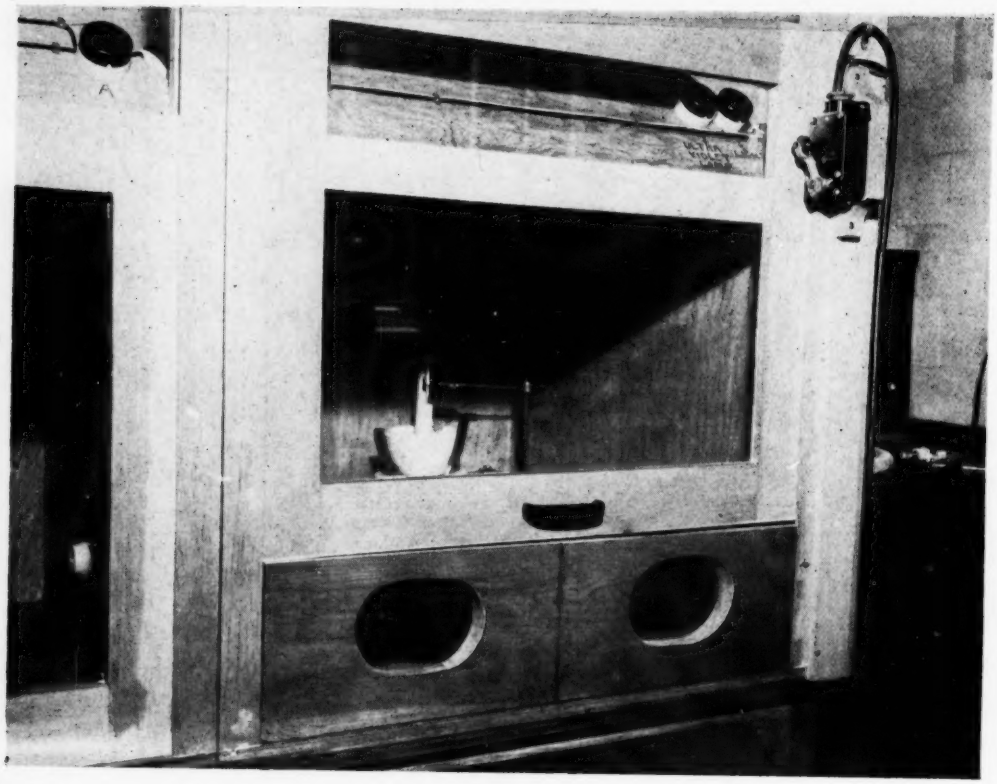


Fig. C.—Exterior view of cabinet with folding panel in place.

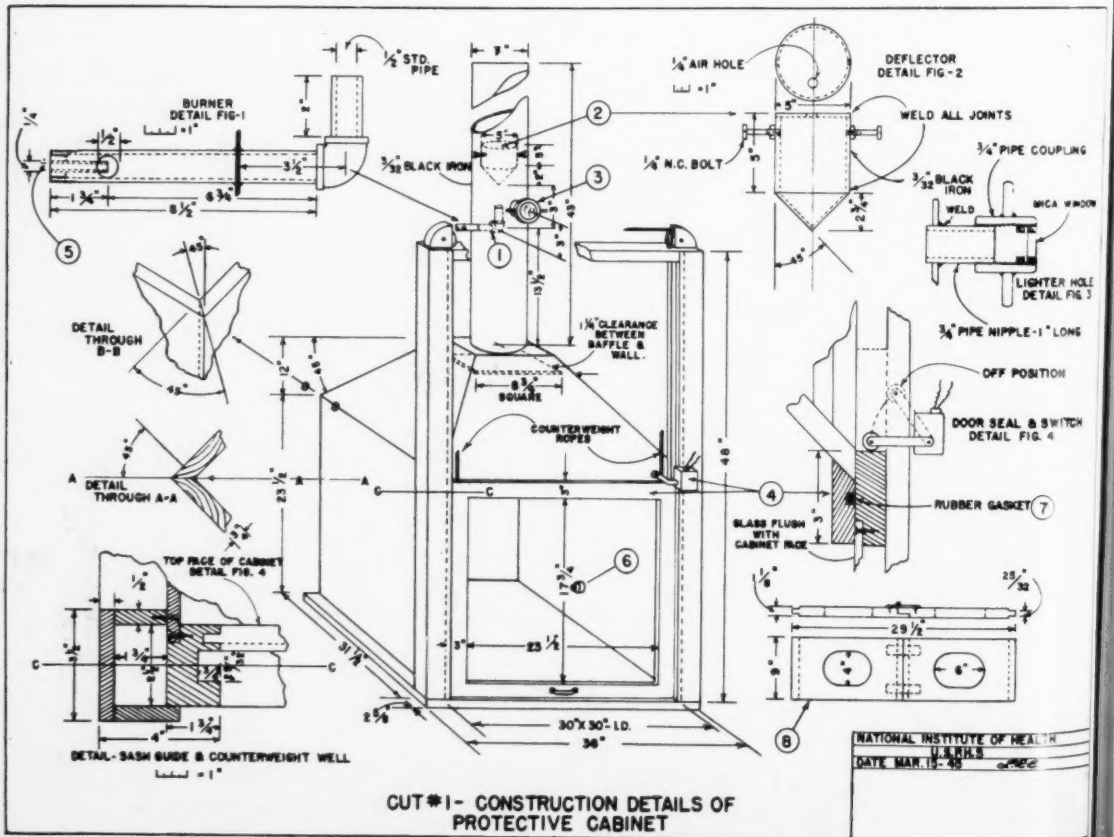


Diagram 1.—Showing construction details of protective cabinet.

Fig. 1), the baffle of $\frac{3}{32}$ inch black iron metal (Diagram 1, *Fig. 2*), and a port for lighting the burner (Diagram 1, *Fig. 3*); and (C) another 19-inch section of galvanized sheet metal stovepipe. The port for lighting the burner contains a small mica window which allows for direct observation of the burner flame.

The method of attaching the electric limit switch is shown in Diagram 1, *Fig. 4*. The switch is so installed that power is supplied to the electric outlets when the window is closed. When the window is open more than $\frac{1}{4}$ inch, the current to these outlets is automatically cut off. The glass in the window must be set flush with the rear side of sash (Diagram 1, *Figs. 4* and *7*) so that it always is in contact with top front panel of cabinet regardless of elevation of the window.

When a hinged panel with armholes (Fig. C and Diagram 1, *Fig. 8*) is provided, the cabinets may be used for a variety of procedures. The electric outlets are not operable when the panel is in place since the limit switch will have broken the circuit. The ultraviolet lamp and the illumination light are operative, however, since their snap switches are independent of the limit switch.

Technique of Operation.—Before the cabinet is used, the gas burner is lighted by putting a burning applicator stick through the port. The gas is turned on slowly until it has ignited and then turned completely on. The burner is allowed to run for from five to ten minutes so that the stack becomes well heated and pulls a good draft. In six minutes the temperature of the air in the stack reaches 500° F. The cabinet may then be used. In order to prevent ultraviolet burns, the bactericidal lamp is kept off while the window is open.

It has been found that, after *B. prodigiosus* was heavily sprayed inside the cabinet, a few colonies developed on agar plates exposed even ten minutes later. This test was done with the burner on and the window closed but with the sterile lamp off. Therefore, in order to clear the box of infectious material in the air, time is allowed for the air to be changed. After use of the blender the window is kept closed for three minutes and then raised an inch or two for twelve minutes, before the blender unit is removed. Raising the window several inches does not destroy the draft.

The panel with armholes is used for handwork which might be dangerous. The size of the holes is not great enough to break the draft. With the board in place various operations may be performed. For example, the opening of bottles containing infectious yolk sacs which have been kept in a cold box will occasionally result in a spray of yolk sac material into the air, and we now prefer to open the bottles in the cabinet. Any handling of yolk sacs which involves possibilities of flipping material into the air may be done inside the box.

When an angle centrifuge is run in the cabinet, there is considerable heating of the air and of the centrifuged material. However, if a wire basket filled with lumps of carbon dioxide ice is placed inside the cabinet, the temperature may be kept at or below room temperature. A six-inch basket full lasts about an hour. On one occasion the burner went out when this procedure was being followed, and we have since raised the window about an eighth of an inch when carbon dioxide is inside.

The stacks on the boxes open into the room, and an exhaust fan some distance from the box removes the hot air to the outside. We have not run the stacks to the outside because of the fear that sudden gusts of wind might

blow back, throwing air into the room and perhaps extinguishing the flame. The procedure has so far been satisfactory. Air leaving the top of the stack appears to be sterile, and no *B. prodigiosus* has been cultured from this air even after heavy spraying of a culture within the cabinet.

One of the chief difficulties met with while the cabinets were being developed was getting sufficient draft. The burner must be sufficiently large, the stack high enough, and the cabinet reasonably tight so that a negative pressure develops inside the cabinet. When these qualifications are met, air is being sucked into the box at all points and passed into the room only after being incinerated. The suction with this design is sufficient to hold flat sheets of paper against the armholes in the folding panel.

SUMMARY

A protective cabinet is described for use in certain procedures in laboratory investigation of infectious diseases. It houses equipment such as tissue grinders and centrifuges which might spray dangerous material in the air. A stack at the top of the box contains a burner which not only incinerates the air, but also reduces pressure inside the cabinet so that all air leaving the box passes through the burner.

DOUBLE-TUBE METHOD FOR ISOLATION OF MOTILE BACTERIA

LIEUTENANT COLONEL MAX LEVINE AND MAJOR PAUL W. PREISLER
SANITARY CORPS, ARMY OF THE UNITED STATES

A SIMPLE double-tube procedure to accomplish the separation of motile from nonmotile bacteria has been devised and is described herewith. The apparatus (see Fig. 1) consists of a standard culture tube of any convenient size (say, 18 to 25 mm. in diameter) containing a small tube (about 75 mm. long and open at both ends) which is provided with a hole (about 3 mm. diameter) in its side at a point about 20 mm. from one end and a 2 to 3 mm. shoulder or ridge which encircles the inner tube about 10 mm. from the other end. The function of this shoulder is to prevent agar from the outer tube passing by capillarity to the rim of, and spreading over into, the inner tube, thereby forming an agar bridge over which bacteria might pass. The small open tube is inserted shoulder end up into the outer tube which is then filled with the culture medium (nutrient broth containing 0.5 per cent agar or other suitable semisolid motility medium) to a point a little below the shoulder of the inner tube and sterilized (or an appropriate quantity of sterilized medium may be added to a sterile double tube). Inoculation is made by stabbing into the agar of the inner tube to a point not closer than about 12 to 15 mm. above the hole in its side.

On incubation, growth of the nonmotile bacteria is restricted to the line of inoculation within the inner tube, whereas motile strains migrate down through the medium of the inner tube and pass through the hole in its side into the outer tube where they disperse throughout the medium from which they can be

Received for publication, May 24, 1945.

fished in the routine manner. By adjusting the distance between the point of inoculation and the hole in the side of the inner tube, separation of actively from sluggishly motile strains may be accomplished.

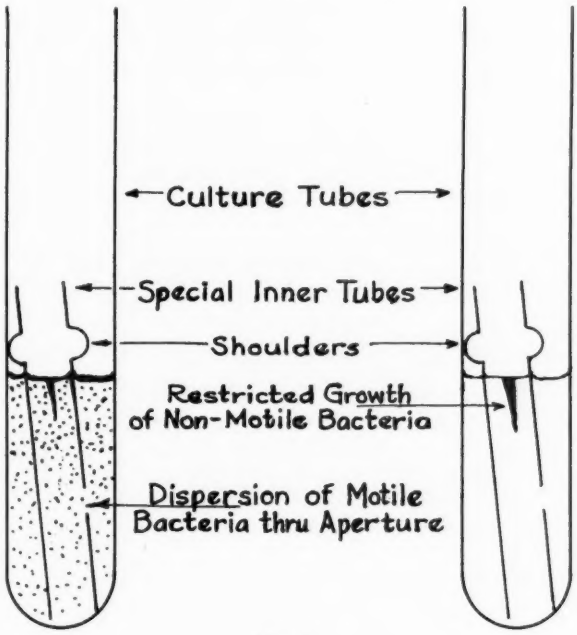


Fig. 1.

This double-tube technique may be employed for isolation of monophasic from diphasic cultures of the Salmonella group by incorporating appropriate antisera into the medium shortly before solidification as suggested by Gard,[1] Edwards and Bruner,[2] and Hajna.[3]

REFERENCES

1. Gard, S.: Ein Colistamm mit Salmonella-H-Antigen, zugleich ein Beitrag zur Frage der Definition der Salmonellagruppe, Ztschr. f. Hyg. u. Infektionskr. **120**: 59, 1937.
2. Edwards, P. R., and Bruner, D. W.: Serological Identification of Salmonella Cultures, Circular 54, Agricultural Experimental Station, University of Kentucky, 1942.
3. Hajna, A. A.: Use of a "U" Tube for the Isolation of Monophasic Varieties From Diphasic Salmonella Cultures, J. Bact. **48**: 609, 1944.

DETERMINATION OF AMINO NITROGEN OF BLOOD FILTRATES BY THE COPPER METHOD

ANTHONY A. ALBANESE, PH.D.,* AND VIRGINIA IRBY, B.S.
BALTIMORE, MD.

IN A PREVIOUS publication[1] which described the adaptation of the copper amino N method to the human urine, it was stated that due to sample size limitations the procedure could not be applied to blood filtrates. Experiments since then, however, have shown that by a suitable adjustment of reagent volumes and concentrations the copper technique can also be employed to advantage in the determination of human plasma or serum amino N. In these biologic fluids, as in the urine, it has been found that other N compounds, namely, creatine creatinine, uric acid, urea, and ammonia, do not participate in the copper reaction, so that it is possible to carry out the estimation without previous manipulation of the blood filtrate. This factor and the microquantitative characteristics of the reaction have been found to permit the accurate performance of this determination with a minimum expenditure of time and apparatus. The results obtained by the method compare favorably with those obtained with other techniques.

EXPERIMENTAL

Reagents.—

Cupric chloride . 2 H_2O: 27.3 Gm. are dissolved in 1 liter of water.

Trisodium phosphate: 64.5 Gm. are dissolved in 1 liter of water.

Borate buffer: To 57.21 Gm. of sodium borate in 1.5 liters of water contained in a 2 liter volumetric flask are added 100 c.c. of N HCl and the volume adjusted to the mark with water.

Copper phosphate suspension: 1 volume of cupric chloride solution is mixed with 2 volumes of trisodium phosphate solution and 2 volumes of borate buffer. This suspension keeps well in the refigerator for about one week.

Thymolphthalein indicator: 0.25 Gm. of thymolphthalein (La Motte) is dissolved in 50 c.c. of 95 per cent ethanol and made to 100 c.c. with water.

Sodium thiosulfate: 49.6 Gm. (0.1 N) are dissolved in 200 c.c. of water and this solution is made to 2 liters after the addition of 20 c.c. of amyl alcohol. The amyl alcohol serves as a stabilizing agent. A 0.001 N solution is prepared and standardized as needed from the 0.1 N stock solution.

Potassium iodate standard: 356.7 mg. (0.01 N) of potassium iodate dried at 110° C. for one hour are dissolved in 1 liter of water; 1 c.c. samples of this solution are used to standardize the thiosulfate solution.

Starch indicator: 1 Gm. of Lintner solublized starch is dissolved in 100 c.c. of saturated NaCl solution by heating on the steam bath.

Potassium iodide: Made as needed by dissolving 1 Gm. of the salt per 1 c.c. of water.

Trichloroacetic acid: 10 per cent solution.

Glacial acetic acid and *1 N. NaOH.*

From the Harriet Lane Home of the Johns Hopkins Hospital and the Department of Pediatrics, The Johns Hopkins University.

Aided by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc.

Received for publication, April 11, 1945.

*Present address: Department of Pediatrics, New York University, Bellevue Hospital, New York, N. Y.

PROCEDURE

Collection of Sample.—Two cubic centimeters of plasma or serum provide sufficient material for duplicate analyses. The plasma may be derived from blood collected over lithium oxalate or sodium citrate in the usual manner. The need for performing the estimation as soon after withdrawal of the blood as possible from the patient cannot be overemphasized. Hemolysis of the sample must also be avoided.

Method.—One cubic centimeter aliquots of plasma or serum are transferred by pipette to 15 c.c. conical centrifuge tubes containing 3 c.c. of 10 per cent trichloroacetic acid. After thorough mixing and standing for ten minutes, the tubes are centrifuged for five minutes at 3,000 r.p.m. The supernatant solutions are decanted through 2.5 cm. funnels containing paper filters (Whatman No. 5) into 100 by 13 mm. tubes and 2 c.c. aliquots are removed to graduated 15 c.c. conical centrifuge tubes. To each of these filtrates is added in succession a drop of thymolyphthalein indicator, N. NaOH to the appearance of the blue color, 5 c.c. of copper phosphate suspension, and distilled water to the 10 c.c. mark. After being mixed by vigorous shaking, the reaction mixtures are allowed to stand for five minutes and then centrifuged for five minutes at 3,000 r.p.m. Five cubic centimeter aliquots of the supernatant fluid are removed from each tube to a 125 c.c. Erlenmeyer flask and acidified with 0.5 c.c. of glacial acetic acid. After the addition of 1 c.c. of KI solution and 3 drops of starch indicator, these samples are titrated with 0.001 N thiosulfate from a very fine-tipped 5 c.c. microburette. The end point is distinct and permanent.

Calculations.—Since each cubic centimeter of 0.001 N thiosulfate is equivalent to 0.028 mg. of amino N, the milligrams of amino N per 100 c.c. of plasma or serum are found from the following formula:

$$\text{c.c. of 0.001 N thiosulfate required for 5 c.c. of final filtrate} \times 0.028 \times 400$$

The agreement between duplicate determinations of the same sample is better than ± 1 per cent.

RESULTS

Representative data from experiments designed to test the applicability of the method to blood filtrates by the recovery of amino N from added amino acids and non-α-amino nitrogenous compounds are given in Table I. For this investigation accurately weighed quantities of chemically pure compounds were dissolved in 10 per cent trichloroacetic acid and 3 c.c. aliquots of these solutions

TABLE I. RECOVERY OF AMINO N OF SUBSTANCES ADDED TO HUMAN PLASMA (1 C.C. OF POOLED HUMAN PLASMA, FOUND TO CONTAIN 0.0694 MG. OF AMINO N PER CUBIC CENTIMETER, USED FOR EACH TEST)

SUBSTANCES	NITROGEN OF COMPOUND ADDED		TOTAL AMINO N IN SAMPLE ANALYZED		RECOVERY OF ADDED AMINO N (PER CENT)
	TOTAL N (MG.)	AMINO N (MG.)	CALCULATED (MG.)	FOUND (MG.)	
Urea	1.40	0	0.0694	0.0689	99.3
Ammonium sulfate	0.93	0	0.0694	0.0708	101.5
Creatine	0.26	0	0.0694	0.0694	100.0
Creatinine	0.45	0	0.0694	0.0694	100.0
Uric acid	0.30	0	0.0694	0.0698	100.5
l (+) Argenine . HCl	1.420	0.355	0.4244	0.4260	100.9
dl-Lysine . HCl	0.698	0.348	0.4174	0.4180	100.2
dl-Alanine	0.972	0.972	1.0414	1.0250	98.5
dl-Methionine	0.563	0.563	0.6324	0.6230	98.6
l (−) Tryptophane	0.854	0.426	0.4954	0.4910	99.5
Amigen*	1.390	0.900	0.9694	0.9740	100.5

*Kindly supplied by Mead Johnson & Co.; an enzymatic digest of casein containing 64.8 per cent of total N as free amino N.

were then employed as the protein precipitants for duplicate 1 c.c. portions of pooled plasma samples. Thus, it was found, as with the urine, that the non-amino substances do not participate in the copper reaction, whereas the amino acids uniformily yield values which are in quantitative accord with their amino N characteristics for the copper reaction. These findings further indicate that no appreciable loss of amino acids by adsorption is incurred from the trichloroacetic acid precipitation of the proteins.

TABLE II. AMINO NITROGEN CONTENT OF ADULT HUMAN BLOOD PLASMA AND SERUM*
(MG. PER 100 C.C.)

NORMAL		ABNORMAL		
DONOR	AMINO N	PATIENT	DIAGNOSIS	AMINO N
A	7.64†	11	Cholecystitis	7.03
B	8.91	17	Nephrosis	7.33
I	5.78	19	Syphilis	7.51†
J	7.84	20	Syphilis	7.57
K	7.72	21	Diabetes	8.12
L	7.72	25	Diabetes	7.71†
M	7.94†	32	Pneumonia	7.92
N	7.48	40	Diabetes	7.36
O	8.28	42	Nephritis	8.96†
P	8.16†	43	Anemia	8.24
Q	8.62	46	Malaria	7.06
R	8.52			
S	8.40			
Average	7.94			7.71
Average deviation	±0.52			±0.36

*Samples marked with dagger (†) were derived from sera; the remainder from plasmas.

The results of the analyses of normal and abnormal human blood plasmas and sera are given in Table II. The blood collections were all made from fasting subjects. A statistical treatment of the data from this limited series shows no significant differences between the plasmas and sera or normals and pathologic entities listed. In one instance, 1 c.c. aliquots of whole blood were analyzed and the amino N value found to be 16 per cent higher than that of the plasma derived from the same blood sample. In this connection, it is of interest to note that eight plasma and serum samples which showed varying degrees of hemolysis yielded an average amino N content of 9.67 ± 0.42 mg. per cent. It is obvious from these observations that hemolysis of the sample must be avoided to obtain an accurate measurement of the plasma or serum amino N.

TABLE III. COMPARISON OF RESULTS OF AMINO N ANALYSES OF HUMAN PLASMA BY THE COPPER METHOD AND OTHER METHODS (MG. PER 100 C.C. PLASMA)

INVESTIGATORS	METHOD	NUMBER OF CASES	AMINO N
Authors	Copper-amino	17	5.8- 8.9
Hamilton and Van Slyke[2]	Ninhydrin—CO_2	13	3.4- 5.5
Cramer and Winnick[3]	Ninhydrin—CO_2	20	2.3- 7.3
Kirk[4]	Gasometric	22	4.5- 8.1
Van Slyke and Kirk[5]	Colorimetric	9	4.8- 7.8
	Gasometric	9	7.5-15.7
	Acetone titration	9	7.2-15.3
	Formol titration	3	10.8-15.0

DISCUSSION

A summary of the data on amino N content of human plasma as reported by various investigators and the present authors is given in Table III. In general, the results of the various methods seem to agree as closely as could be expected considering the fact that the different methods do not determine exactly the same amount of amino N in each amino acid. The greatest differ-

ence is found in some of the high values secured by Van Slyke and Kirk[5] with the gasometric, acetone titration, and formol titration techniques, but these may have been due to some peculiarity of the unreported condition of the patients.

From the observations of others[6] and our own short series, the measurement of amino N level of the plasma appears to be of only limited diagnostic value. However, it is a valuable index in the study of the intermediary metabolism of amino acids and related physiologic experiments. In our experience the method described is ideally suited for investigations which demand numerous determinations, in that the procedure does not require the time-consuming preliminary treatment of the samples for the removal of urea, ammonia, or CO_2 which is the case with the other available methods. An idea of the rapidity of operation of the copper amino N technique can be gained from the fact that a trained assistant can perform from ten to twelve analyses within two hours with an accuracy of better than 1 per cent.

SUMMARY

The copper amino N method has been successfully adapted for use with 1 c.c. of human serum or plasma. Analysis of the blood of thirteen normal subjects yielded an amino N value of 7.94 ± 0.52 mg. per cent; and eleven abnormal cases, 7.71 ± 0.76 mg. per cent. These data are compared with those obtained by other investigators employing the available procedures.

REFERENCES

1. (a) Albanese, A. A., and Irby, V.: Determination of Urinary Amino Nitrogen by the Copper Method, J. Biol. Chem. **153**: 583, 1944.
   (b) Pope, C. G., and Stevens, M. F.: The Determination of Amino-Nitrogen Using a Copper Method, Biochem. J. **33**: 1070, 1939.
2. Hamilton, P. B., and Van Slyke, D. D.: The Gasometric Determination of Free Amino Acids in Blood Filtrates by the Ninhydrin-Carbon Dioxide Method, J. Biol. Chem. **150**: 231, 1943.
3. Cramer, L. B., and Winnick, T.: Amino Acid Nitrogen of Normal Human Plasma, J. Biol. Chem. **150**: 259, 1943.
4. Kirk, E.: Amino Acid and Ammonia Metabolism in Liver Diseases, Acta. Med. Scandinav., supp. 77, pp. 1-147, 1936.
5. Van Slyke, D. D., and Kirk, E.: Comparison of Gasometric, Colorimetric, and Titrimetric Determinations of Amino Nitrogen in Blood and Urine, J. Biol. Chem. **102**: 651, 1933.
6. Desqueyroux, J.: Recherches cliniques sur l'amino-acidemia, Ann. de méd. **13**: 20, 1923.
7. Greene, C. H., Sandiford, K., and Ross, H.: The Amino Acid Content of the Blood in Normal and Pathologic Conditions, J. Biol. Chem. **58**: 845, 1924.
8. Feinblatt, H. M., and Shapiro, I.: The Amino Acid Content of Blood in Various Pathologic Conditions, Arch. Int. Med. **34**: 690, 1924.
9. Edgar, S. H.: The Amino Acid Content of the Blood of Children in Health and Disease, Biochem. J. **22**: 168, 1928.

BOOK REVIEWS

A Manual of Tropical Medicine. By *Colonel Thomas T. Mackie*, M.C., A.U.S. Executive Officer, Tropical and Military Medicine; Chief, Division of Parasitology, Army Medical School; *Major George W. Hunter, III*, Sn.C., A.U.S., Division of Parasitology, Army Medical School; and *Captain C. Brooke Worth*, M.C., A.U.S., Division of Parasitology, Army Medical School. W. B. Saunders Company, Philadelphia, Pa. Price $6.00. Cloth with 727 pages and 287 illustrations.

The authors have succeeded in condensing salient material and much information of practical value into a concise manual of tropical medicine. The material is presented briefly yet in sufficient detail to be completely understandable. The illustrations are particularly good. The charts depicting the epidemiology of various diseases are of considerable interest and serve to emphasize important features in the control of disease. Therapeutic measures are covered adequately.

Section X, under the heading Medically Important Arthropods, stresses the tremendous importance of the arthropod in world disease, 142 pages being devoted to entomology. Unnecessary biological details are omitted, yet the subject is well covered, showing clearly the role of the arthropod in disease processes. Table 49, *Human Disease Transmitted by Arthropods*, is an excellent summary of present-day knowledge on the subject. The entomological illustrations are good and should be of help to the novice who requires a working knowledge of this difficult and vast field.

The importance of laboratory procedures in the diagnosis of tropical disease is obvious. The authors have presented in Section XI, *Laboratory Diagnostic Methods*, a concise and clearly written outline of useful laboratory information. This portion of the manual should be especially helpful to the general practicing clinician who may not have adequate laboratory facilities at his disposal.

For military and civilian physicians *A Manual of Tropical Medicine* will serve as a useful and reliable guide through the welter of subjects included under the heading Tropical Medicine. For the medical student, the simple, clear, and concise presentation of material will be of assistance in the perusal of more detailed volumes on tropical medicine.

R. J. B.

Penicillin Therapy Including Tyrothricin and Other Antibiotic Therapy. By *John A. Kolmer*, M.S., M.D., Dr. P.H., Sc.D., LL.D., L.H.D., F.A.C.P. Professor of Medicine in the School of Medicine and the School of Dentistry, Temple University; Director of the Research Institute of Cutaneous Medicine; Formerly Professor of Pathology and Bacteriology, Graduate School of Medicine, University of Pennsylvania. D. Appleton-Century Company, Inc., 1945, New York, N. Y. Price $5.00. Cloth with 302 pages.

The literature on penicillin has become extensive, as is always the case with any strikingly successful clinical procedure or agent. At the present time penicillin may be obtained by medical practitioners generally, so that the need for correlating and summarizing the knowledge about it for practical purposes is evident. In this book Dr. Kolmer has prepared an extensive review of the literature with references to most of the original work and has made a particular effort to give the details needed for clinical use of the drug. It seems to this reviewer that this is worth while and important but that he has gone too far in two respects. In his desire to include all possible details, he has given many that are unnecessary, such as a full-page illustration of a nurse injecting fluid into a vial with a syringe. Also, the numerous possible applications of penicillin therapy make a very large number of variations in dosage and methods of administration and there are often minor differences of opinion on these matters in individual situations. Dr. Kolmer has covered these points in a detailed and encyclopedic manner and I think has not emphasized sufficiently that the intelligent use of the drug must depend primarily on a knowledge of the fundamental principles of action and pathogenesis rather than on the exact procedure used by someone else in the same disease. A few important references have been omitted from the book, but on the whole it is valuable and may be recommended.

C. G. Harford.

Penicillin and Other Antibiotic Agents. By *Wallace E. Herrell,* M.D., M.S., F.A.C.P. Assistant Professor of Medicine, The Mayo Foundation, University of Minnesota; Consultant in Medicine, Mayo Clinic, Rochester, Minn. W. B. Saunders Co., Philadelphia, Pa. Price $5.00. Cloth with 348 pages and 45 illustrations.

Perhaps no therapeutic agent was more critically evaluated in such a short period of time than was penicillin. This does not apply to the other antibiotics with the possible exception of tyrothricin. Intense interest has been manifested by the medical profession in the use of these agents, especially penicillin. In view of this, Dr. Herrell has attempted to crystallize the experimental and clinical studies carried out with penicillin and other agents. This is an aspiration of considerable magnitude.

The monograph is divided into four parts. Part I is an exposition of the well-known history of penicillin; its properties; pharmacology; and assay methods. In Parts II and III are detailed the clinical use of penicillin and its toxicity. In Part IV are discussed other antibiotic agents such as tyrothricin, streptothricin, and streptomycin. Chapters within each of these main sections are followed by references to the literature on the subject.

The text is well written and edited. It is not surprising that a definitive statement is not made concerning many aspects of the subject, such as the chemistry of penicillin and its mode of action, and the clinical use of streptomycin. Likewise, after reading the clinical sections, the physician will be left in a quandary as to the value of penicillin in such diseases as scarlet fever and peritonitis. Nevertheless, most of the information available has been included in this work. The section on tyrothricin is particularly well done.

The publishers are to be commended for the excellency of the print and reproduction of illustrations.

W. W. S.

Microbial Antagonisms and Antibiotic Substances. By *Selman A. Waksman,* Professor of Microbiology, Rutgers University; Microbiologist, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey. The Commonwealth Fund, New York, N. Y. Price $3.75. Cloth with 350 pages.

The recent discovery of the therapeutic value of certain antibiotic substances has resulted in a great wave of interest in the material dealt with in this book. As the title indicates, it is more than a treatise on antibiotic agents. Doctor Waksman has long been a leading figure in the field of soil microbiology, and since the soil is the source of most organisms known to produce antibiotic substances, it is not mere chance that he is now a leader in this new field. The book opens with a general discussion of soil and water as habitats of microorganisms. This discussion, dealing with the interrelationships between organisms in the soil, the physical and chemical nature of the medium, the fate of pathogens on introduction into the soil, pathogens of soil origin, etc., should prove very interesting and instructive to most bacteriologists and medical men, as well as other biologists who have been trained to think in terms of pure cultures and single infections instead of organisms in their natural habitats. Special attention is given to the subject of plant and animal wastes as related to soil populations. In bringing the reader closer to the major thesis of the book, such phenomena as synergism, symbiosis, parasitism, and antibiosis are discussed.

The fourth chapter deals with the more practical problems of isolation and cultivation of antagonistic organisms and the measurement of antibiotic action. The next several chapters are devoted to bacteria, actinomycetes, fungi, and animal forms as antagonists. In completing this aspect of the subject, a short chapter is devoted to the antagonistic relationships between bacteria and viruses and among viruses themselves.

The second half of the volume deals with more familiar subjects such as the chemical nature of antibiotic substances, the mechanism of antibiotic action, and disease control. In the final chapter Doctor Waksman speculates a bit on the future. No definite promises are made, but he leaves no doubt in the reader's mind that this is a field of science in which much may be expected. Already the problems have far outgrown the limits of microbiology, and as time goes on, the chemist, the chemotherapist, the physiologist, and other specialists will make substantial contributions in the development of this field.

This book is exceptionally well documented, having a bibliography of more than 1,000 references. Two extensive indexes are provided; one lists the microorganisms mentioned and the other covers the general subject matter. The work is very readable and should prove of much interest and value to all students of biology and medicine, and particularly to workers interested in the special problems of antibiosis.

J. Arthur Herrick.

Handbook of Practical Bacteriology. By *T. J. Mackie*, C.B.E., M.D., D.P.H. Professor of Bacteriology, University of Edinburgh; Honorary Bacteriologist to the Royal Infirmary, Edinburgh; Director of Bacteriological Service, City of Edinburgh; Regional Director S.E. Scotland, Emergency Bacteriological Service; and J. M. McCartney, M.D., D.Sc., Director of Research and Pathological Service, London County Council; Major, R.A.M.C.; formerly Lecturer in Bacteriology, University of Edinburgh; Fellow of the Rockefeller Institute for Medical Research, N. Y. Seventh Edition. Williams & Wilkins Company, Baltimore Md. Price $5.00. Cloth with 720 pages.

Diagnostic Procedures and Reagents. Second Edition. Edited by the American Public Health Association, New York, N. Y. Price $4.00. Cloth with 549 pages.

The Male Hormone. By *Paul DeKruif*. Harcourt, Brace and Company, New York, N. Y. Price $2.50. Cloth with 243 pages.